THE CHARACTERS AND DISTRIBUTION OF THE COLIFORM GROUP OF BACTERIA

A detailed and critical study of the literature, having particular reference to the significance of these bacteria in the examination of water supplies, and including the results of some short experiments bearing upon the subject.

by

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FOREWORD.

An eminent psychologist has achieved considerable notoriety in recent years by replying to many different questions put to him: "It all depends on what you mean by 'so-and-so' and what you mean by 'such-and-such'." However justified may have been these Irish answers in the spheres in which they were given, I submit that this same gentleman, had he been a bacteriologist, could with at least equal justification have answered many questions regarding the coliform group of bacteria by saying: "It all depends on what you mean by 'B. coli'." The confusion arising from the necessarily inadequate definition of 'B. coli' by the earlier investigators has persisted in some degree to the present day, so that even now it is necessary to distinguish between:

(1) B. coli - loosely applied to mean the entire coliform group and/or any of its members.

(2) B. coli - even more loosely extended (e.g., "presumptive B. coli") to include any micro-organism - sporing or otherwise - or mixture of organisms, which can produce acid and gas when grown in a liquid lactose medium.

(3) B. coli - properly (in my opinion) applied to mean the definite sub-group termed "Bact. coli, type I, faecal" by the Ministry of Health (1939)

(4) B. coli - still more restricted to apply only to the type species, i.e., B. coli communis (Escherich).

If, therefore, in the ensuing pages the emphasis on exact definitions becomes tedious, may it be remembered that final decisions have not yet been reached regarding the importance of certain differences between the various types, and that what may to-day seem relatively trivial (as, for example, the Voges-Proskauer reaction in Mac-Conkey's time) may later prove (as the Voges-Proskauer reaction has done) to be fundamental.

As quite a number of the tables presented in the following pages have made unavoidable encroachments on both margins, it is respectfully suggested that the outside cover be removed before the work is read.

The Characters and Distribution of the Coliform Bacteria.

EARLY HISTORY AND LITERATURE

Period 1885 - 1905

The story of the coliform group dates back to 1885 when Escherich, in investigating the bacterial flora of milk-fed infants' faeces, described the occurrence therein of two types of Gram-negative, rod-shaped, non-sporing organisms which produced gas when grown in a glucose medium and gave rise to (what he considered) characteristic colonies on gelatin, agar and potato. One type, to which he gave the name of Bacillus coli communis, consisted of relatively long, motile rods which when grown in milk slowly produced coagulation. The other type tended to form shorter and thicker rods which were non-motile and coagulated milk more vigorously. He called this variety Bacillus lactis aerogenes.

A motile strain, culturally akin to B. lactis aerogenes except that it liquefied the gelatin medium, was subsequently described by Jordan (1890) who recovered it from sewage, and named it B. cloacae.

In 1894, Dyer and Keith, using as their criteria cultural characters on agar, gelatin and potato, reduction of nitrates and clotting of milk, reported a type of organism - indistinguishable from the human B. coli - to be normally present in the faeces of goats, rabbits, pigs and other domestic animals. Kruse (1894) showed that organisms resembling B. coli were not only widely distributed as intestinal parasites but were also to be found in water and soil. He called attention to their heterogeneity and made the prophetic statement (to be more and more fully realised in later years) that the organisms covered by the term "B. coli" represented not one single type but a whole group of closely related species.

Theobald Smith (1891,1895) had meanwhile noticed that B. lactis aerogenes and B. cloacae produced more gas from glucose than did B. coli. He showed that in all cases the gas consisted of hydrogen and carbon dioxide, and that not only the total volume but also the proportions of the mixture varied with the different types. His results may be tabulated thus:

	Total Volume	Gas Ratio (CO_2/H_2)
B. coli	50%	1/2
B. lactis aerog.	80-100%	1+/1
B. cloacae	90-100%	2/1 or 3/1

where "100%" represented the total length of the tube.

He added further fermentation tests and defined B. coli as a motile bacillus fermenting glucose, lactose

and sometimes sucrose, producing acid and clot in milk and giving the above gas ratio in glucose. He stressed the value of the gas ratio and total gas volume as differential tests and, together with Brown (1893), had introduced the fermentation tube for approximately enumerating the faecal bacteria in water. These authors made the (now remarkable) statement that "the numbers of B. coli communis found in waters considered on inspection to be unpolluted vary from 9 to 5 (per c.c.)".

Grimbert (1896) and Refik (1896) appear to have been the first to apply the indole test to the coliform group. Grimbert defined B. coli as a motile fermenter of glucose and lactose which had no action on sucrose, but clotted milk and produced indole. He also stated that the property of fermenting sucrose was not usual among the coli group. He believed that the B. pneumoniae (Friedländer) was to be found in water much more frequently than was generally imagined. This organism, by his definition, was a Gram-negative, non-motile, lactose-fermenter which did not liquefy gelatin or produce indole. Theobald Smith had already (1891) reported finding this bacillus in swine faeces. He described it as a bacillus giving a general turbidity in broth, acid and clot in milk, not liquefying gelatin, and fermenting glucose, lactose and sucrose. The gas-ratio (CO_2/H_2) was 1/1+.

Refik (1896) attempted to classify the coliform bacteria which he found in water. He used four tests: the fermentation of glucose and lactose, the clotting of milk and the production of indole. He concluded that there was no correlation between sugar fermentations and the clotting of milk.

Hammerl (1897) studied the effect of food on the bacterial flora in human faeces. He noted that the food, whether sterile or not, whether vegetable or mixed, had little influence on the number of living bacteria in the faeces. He did find, however, that when the food was sterile the usual saprophytes commonly found in man's surroundings disappeared from the faeces and almost only B. coli and B. lactis aerogenes were present.

The CO_2/H_2 gas-ratio had meanwhile been the subject of further research (Pammel and Pammel, 1896; Grimbert, 1896; Harden, 1899, 1901), and Russell and Bassett (1899) confirmed the differential value of a high or low ratio, noting that the high ratio types appeared to be normal soil forms rather than intestinal parasites. Houston (1898, 1901, 1903), having tested numerous samples of virgin soil, stated that B. coli (motility$_{\pm}$, indole$_{+}$, glucose$_{+}$, lactose$_{+}$, sucrose$_{\pm}$, milk$_{AC}$) was either absent from virgin soil or else present in very small numbers.

Laurent (1899) during a study of certain diseases of potato noted the occurrence of a bacillus which proved to be of the colon type - a facultative anaerobe, not liquefying gelatin but able to reduce nitrates, produce a clot in milk, and give acid and gas in glucose and lactose broth. He showed that this organism could become parasitic upon plants which had been devitalised by receiving large amounts of chalk, and upon potatoes whose resistance had been similarly reduced by immersion in an alkaline solution. Klein and Houston (1899-1900) demonstrated the presence of coliform organisms in various cereals (e.g., rice, flour and oatmeal). The tests used were the type of growth in phenol broth, the absence of liquefaction of gelatin, the clotting of milk, the production of indole and the pathogenicity. Only 6 out of 36 organisms isolated proved to be "typical B. coli" though a further 7 failed only on the milk or the gelatin test.

In 1898, Voges and Proskauer, in an enquiry into the cause of haemorrhagic septicaemia, discovered that, when a solution of potassium hydroxide was added to a glucose broth culture of certain intestinal bacilli and the tube then allowed to stand at room temperature, a red fluorescence gradually developed in the upper layer of the fluid which was exposed to the air. While they could not explain the reaction, they submitted several different kinds of bacilli to the test and found that B. coli, among others, failed to produce the colour. This, as regards the classification of the coliform group, was undoubtedly the most fundamental discovery of the 19th century.

The introduction by Durham (1898) of the inverted tube for demonstrating bacterial gas-production marked a general bacteriological advance which was particularly applicable to the coliform group, and in 1901 he published a proposed classification of intestinal bacteria based on sugar fermentations. This may be summarised as follows:-

DIVISION 1. Includes the typhoid bacilli.

DIVISION 2. Motile bacilli of colon-like morphology.

	Dextrose	Lactose	Sucrose	
Order 1.	AG	--	--	colon-like in rate of growth.
Order 2.	AG	AG	--	B. coli communis verus.
Order 3.	AG	AG	AG	B. coli communior.

DIVISION 3. Non-motile polysaccharide fractors, viz: shorter and thicker rods which produce acid and gas not only from dextrose, lactose and sucrose but also from starch and inulin. This group includes B. lactis aerogenes.

Durham suggested that because of its greater relative frequency that form of B. coli which fermented sucrose should be given the name B. coli communior and the B. coli communis (Esherich) - which does not ferment sucrose - should be termed B. coli communis verus. He noted (and this is more important) that all the bacilli in his 3rd division gave a positive Voges-Proskauer test while those in the other groups all gave negative results. Durham had at the same time explored the possibility of employing agglutination for classifying the coliform bacteria, but was forced to dismiss this as useless. By using, however, the five carbohydrates together with the motility, morphology and Voges-Proskauer reaction, he had made a distinct advance and had laid the foundation for the best of MacConkey's subsequent work.

Ford (1901) by post-mortem examination investigated the human intestinal flora at different levels. He used Durham's sugars and distinguished (a) the B. coli, (b) the B. lactis aerogenes, and (c) the B. cloacae groups. He found B. coli communior (Durham) to be the commonest organism and noted that B. coli communis verus (Durham) was also very frequently present. In similar experiments (1903) he reported that about 28% of 200 coliform organisms isolated post-mortem from the human intestine were of the B. lactis aerogenes type. Hellström (1901) examined the faeces of new-born children and found the first meconium was sterile. Later, air organisms appeared, and, later still, these disappeared and were replaced by the usual intestinal organisms, especially B. coli and B. lactis aerogenes. Horrocks (1901) compared 80 strains of coli from typhoid stools with 70 from normal faeces, but could find no constant distinguishing features. Heinick (1903) bacteriologically examined the intestinal canal of 23 pigs. Of the organisms present only B. coli and B. lactis aerogenes occurred regularly. The B. coli were the most numerous but B. lactis aerogenes occurred in almost equal numbers. Houston (1902-3) attempted a numerical estimation of the viable coliform bacteria in normal human faeces and arrived at the conclusion that there were between 100 million and 1000 million "B. coli" per gramme of faeces. He isolated 100 strains and obtained the following results:-

indole_{+}	99%	
milk_{AC}	92%	(8% gave acid only)
dulcite_{AG}	55%	
sucrose_{AG}	30%	(35% ?-ve, 35% -ve)

In 1901, Houston had implanted soil with sewage and subsequently recovered a number of indole-negative, weak-clotting coliforms. He concluded that either the "typical" B. coli died out in soil more rapidly than the atypical coliforms, or else the true coli strains lost some of their properties as the result of prolonged

incubation in soil. Jordan (1901), in an interesting enquiry into the self-purification of the Illinois river (which then received pollution only at Chicago), came to a similar conclusion: "It is quite possible that some colon bacilli may become so disguised by prolonged aquatic life as to be no longer recognisable by the methods used." Tracing the river from Chicago (where the pollution was equivalent to that of raw sewage) he found a gradual improvement which was greater than could be accounted for by dilution. He concluded that this might be due to (1) death and sedimentation of the coliforms which the examination revealed, or (2) modification of the bacterial properties.

Horrocks (1903), on the other hand, contended that B. coli never becomes atypical in water. He planted B. coli communis (Escherich) on various soil samples and recovered it unchanged after 30-60 days. Similarly, from deep well waters he recovered it unaltered after 31 days, though after 9 weeks to 3 months there was weakened indole-production or delayed clotting of milk. Savage (1905) upheld Horrocks' views and later (1907) advanced the opinion that "typical B. coli" implanted into soil showed some alteration of character, but that the changes were not extensive and no evidence was obtained that the widely aberrant organisms met with in different soils and waters ever represented typical B. coli altered by unfavorable environment. MacConkey (1905) questioned whether any variation occurred at all, and devised an ingenious experiment whereby a strain of B. coli communis (Escherich) was retained in a Pasteur candle filter which was immersed in tap water. Though the tap water was changed from time to time, and at intervals removed altogether so that the candle and its contents became dry, the organism was continuously recovered unchanged up to and including the 358th day. His tests, however, were confined to agar, gelatin, broth, litmus milk and the following 'sugars'- glucose, lactose, mannite, sucrose and dulcite. It is unfortunate that the indole test was not included.

The exact significance of B. coli in drinking water had meanwhile been the subject of much conflicting opinion, ranging from (1) a readiness to accept its presence as indicative of serious pollution, irrespective of the number present, to (2) a reluctance to credit it with any faecal significance on the grounds that it could possibly have been derived from innocent sources. In this latter respect, Jordan (1890) had, for instance, objected to B. coli as a true index of sewage contamination because he had found "in spring water, which was beyond any suspicion of contamination, bacteria which in form, size, growth on gelatine, potato, etc. were indistinguishable from Bacillus coli communis." That the problem was a quantitative rather than a qualitative one,

or that both aspects were of importance, became however gradually appreciated, and Pakes (1900) set a series of standards. Deep well waters, he said, should be free from B. coli altogether. Waters from other sources which contained B. coli (1) only in a volume greater than 100 c.c. were probably safe, (2) in a volume of between 50 and 100 c.c. should be regarded as slightly suspicious, (3) in a volume of 20-50 c.c. were suspicious, and (4) in 20 c.c. or less should be condemned. Houston (1901) held similar views, and Savage (1902) did likewise except that he was prepared to allow greater latitude to upland surface waters.

In the period 1900-1905 there follows a number of efforts to define and distinguish some of the coliform strains. Grimbert and Legros (1900) considered that B. lactis aerogenes (Escherich) and B. pneumoniae (Friedländer) were the same organism because they (1) were non-motile, (2) formed capsules in the blood of inoculated animals, (3) did not liquefy gelatin, (4) did not form indole, and (5) fermented similar though not always identical carbohydrates and alcohols. They distinguished the coli group by its motility, capacity to form indole, and inability to form capsules. Rothberger (1900) described B. lactis aerogenes (Escherich) as a non-motile bacillus which did not form indole but produced general turbidity in broth, acid and clot in milk and acid and gas in glucose. Hewlett (1902) - cf. Durham (1901) - regarded the power of fermenting starch and inulin as a characteristic of B. lactis aerogenes. He defined B. pneumoniae (Friedländer) as of variable morphology, non-motile, Gram-negative, indole-negative, not liquefying gelatin, but clotting milk, giving a copious viscid growth on agar, and producing a characteristic "nail-growth" in a gelatin stab culture. He considered that the B. coli communis had been described under the name of B. cavicida (Brieger) and B. neapolitanus (Emmerich).

Muir and Ritchie (1902) believed that B. lactis aerogenes was either a variety of B. coli or a closely related organism. They distinguished it by its more abundant gas production and by its growth on gelatin and agar being thicker and whiter than that of coli. They agreed with Hewlett's description of friedländeri.

Prescott (1903) isolated "lactic acid bacilli" (B. acidi lactici) from various cereals and meals and from waters which were apparently free from all suspicion of faecal contamination, and compared them with 23 varieties of B. coli which had been isolated from faeces or sewage. Of 64 cultures 44 gave the same cultural reactions, and, of these, 25 were lactic acid bacteria and 19 were coli. Morphologically there was no difference and all produced the same amount of acid when tested under the same conditions. Lehmann and Neumann (1904) describe B. acidi lactici as a short, oval, non-motile rod which was Gram-

negative and did not liquefy gelatin. It produced general turbidity in broth, indole in peptone solutions, clotting in milk, and acid and gas in glucose and lactose. They considered it to be identical with B. lactis aerogenes (Escherich) and believed that the B. cavicida (Brieger) and B. neapolitanus (Emmerich) were most closely related to it. B. pneumoniae (Friedländer) they detailed as a non-motile, non-sporing, Gram-negative rod giving round slimy colonies on gelatin. Indole production was slight.

Jordan (1903) in an investigation into the numerous classes of bacteria which he obtained by various methods from river water defined B. coli as a motile organism producing a rapid clot in milk, usually forming indole, not liquefying gelatin, but fermenting glucose and lactose always and sucrose sometimes. B. lactis aerogenes, he considered, had similar characters except for its non-motility, a more "convex and fleshy" growth on gelatin, and a slower clotting of milk. He did not regard the ability to ferment lactose as an essential characteristic of his B. cloacae. This, he stated, was often slow and sometimes absent. Otherwise, his bacillus was motile, Gram-negative, clotted milk, nearly always liquefied gelatin, fermented glucose and sucrose, and often produced indole.

The work of Howe (1904) presents a refreshing change from the above mass of apparent contradiction and confusion. He isolated from water a number of coliform bacteria which had the following characters in common: producing acid and gas in glucose, clotting milk, forming red colonies on litmus lactose agar, producing indole, and reducing nitrates. To these tests he added the Voges-Proskauer reaction and noted that: (1) the reaction was positive only with cultures which produced a large amount of gas in glucose, and (2) more sugar was left in the tubes which gave a negative reaction. He considered that the development of this colour is a perfectly definite distinguishing feature of the bacteria by which it is produced, and suggested that the test be applied to coliform organisms from various sources.

It suffices to add in this section that MacConkey and Hill (1901) introduced bile-salt (sodium taurocholate) as a selective ingredient in media for the isolation of intestinal bacteria. They had found this to be the most effective agent for inhibiting the growth of extraneous bacteria while permitting the intestinal organisms to develop almost as usual. All members of the coliform group grew well in the presence of bile-salt, i.e., were "bile-tolerant", so that a further group characteristic had been established as well as an extremely useful series of media invented. Litmus was then in general use as the "indicator" of acid production. Grünbaum and Hume (1902) advocated its replace-

ment by neutral-red as affording certain technical advantages. It was, therefore, during this period (1900-1905) that the now world-famous neutral-red, bile-salt, lactose, peptone water and solid media ("MacConkey Broth" and "MacConkey Agar") were evolved.

Summary and Discussion
(1885-1905)

It has been shown that Escherich (1885) isolated from human faeces and briefly described two strains of what came to be called coliform bacteria. Similar organisms were soon afterwards recovered from animal faeces, sewage, water, soil and various cereals, but the paucity of the original descriptions left ample scope for ambiguity, and, as each subsequent investigator added his own (or stated no) criteria for "B. coli", "B. lactis aerogenes", etc., considerable confusion resulted. Hence, not a few of the observations made during the period under review require to be accepted with reserve or almost ignored.

In Table 1 an effort has been made to show from how many different stand-points the problem of classification was approached and to what extent current ideas agreed or differed.

The characters common to the whole group received, perhaps, most general agreement. Coliform bacteria were Gram-negative, non-sporing, rod-shaped organisms which grew as aerobes and facultative anaerobes on and in the usual laboratory media at room temperature and blood heat, forming colourless colonies of varying character on gelatin and agar, producing acid and clot in milk, and fermenting glucose and lactose with the production of acid and gas. (Jordan's (1903) conception of his B. cloacae formed the only exception to this general rule, in that lactose-fermentation was not a necessary qualification, though it is difficult to conceive how otherwise milk was always clotted.) Properties which were variously present in different strains and which were, therefore, used as distinguishing features were the morphology, motility, colony characters, growth in broth and in gelatin stabs, liquefaction of gelatin, fermentation of sucrose, starch and inulin, production of indole, the amount of gas produced from glucose, the CO_2/H_2 ratio, the Voges-Proskauer reaction and the reduction of nitrates.

Of the different types, B. coli (type species: B. coli communis, Escherich) was the best defined. It was a slender, generally motile rod which did not liquefy gelatin, fermented sucrose sometimes, failed to attack starch and inulin, produced indole (usually, according

TABLE 1.

Characters ascribed to the early Coliform Types (1885-1904)

Reference	ORGANISM	Motility	Capsule-formn.	Colonies on gelatin, agar, etc.	Liquefaction (gelⁿ.)	Clotting of milk	Prodⁿ. of acid and gas in: Glucose	Lactose	Sucrose	Starch	Inulin	Indole-production	CO_2/H_2 ratio	V.P. reaction	Reduction of nitrates	Source (if any)
Escherich 1885	B. coli (typical)	+		typ.[1]		+	+									Human faeces
Dyer and Keith 1894		+		typ.[1]		+	+								+	Animal faeces
T. Smith 1895		+				+	+	+					[1]			
Grimbert 1896		+				+	+	+	-	+						
Voges & Prosr 1898		+			-	+	+	+				+		-		Intestine
Laurent 1899		+			-	+	+	+							+	Potato
Grimbert & Legros 1900		+	-				+	+				+				
Durham 1901		+				+	+	+	±	-				-		Intestine
Houston 1901		+				+	+	+	±		-					Not virgin soil
Jordan 1903		+			-	+	+	+	±			(±)				River water
Escherich 1885	B. lactis aerogenes	-		typ.[1]		+	+									Human faeces
Grim.&Legros 1900		-	+		-		+	+				-				
Rothberger 1900		-				+	+									
Durham 1901		-				+	+	+	+	(+)	(+)	-		+		Intestine
Hewlett 1902		-								+	+					
Jordan 1903		-		fleshy	-	+	+	+	+			(±)				River water
Lehmann & Neumn. 1904		-			-	+	+	+				+				
Jordan	B. cloacae	+			(±)	+	+									Sewage
Jordan 1903		+			(±)	+	+	+	+			(±)				River water
T. Smith 1891	B. pneumoniae (Friedländer)	-			-	+	+	+	+				<1			Water
Grimbert 1896		-			-			+				-				
Grim.&Legros 1900		-	+		-		+	+								
Hewlett 1902		-		viscid	-							-				
Muir & Ritchie 1902																
Lehmann&Neumann 1904		-		slimy								sl+				
Lehmann & Neumann 1904	B. acidi lactici	-			-	+	+	+				+				

+ = positive (production of acid and gas, etc.)
(+) = usually positive; ± = positive or negative

to Jordan), gave a low gas-ratio and a negative Voges-Proskauer reaction, and reduced nitrates.

B. lactis aerogenes was a thicker rod, invariably non-motile, producing thicker and whiter colonies on gelatin which it failed to liquefy, and usually fermenting sucrose and (by some authorities) also starch and inulin. Opinion was divided regarding indole-production, but (excluding misinterpretations now obvious) the gas-ratio was constantly high (i.e. greater than 1) and the Voges-Proskauer reaction positive.

The original characteristics of Jordan's B. cloacae were its motility and liquefying power. Recognising it on this basis, Smith (1895) showed it to be a high gas-ratio type. It would appear, however, that Jordan had been meanwhile extending his definition, for by 1903 he had included under this term motile fermenters of glucose and sucrose which failed to liquefy gelatin and might not even ferment lactose. (He had, indeed, classed cloacae as a sub-group of Proteus.) So far as cloacae is concerned, Jordan's views must receive attention, but little weight can be attached to his idea of B. lactis aerogenes, for he not only reverses Escherich's distinction on activity of clotting but treats aerogenes almost as if it were merely a non-motile form of coli, a misconception which he shared with T. Smith (1895), Muir and Ritchie (1902), Kruse (1903), and Lehmann and Neumann (1904).

B. pneumoniae (Friedländer) was distinguished quite generally as a non-motile organism which produced viscid or slimy colonies, did not liquefy gelatin, but fermented sucrose (Smith, 1895), did not form indole (or just feebly so - Lehmann and Neumann, 1904), and produced a low gas-ratio.

B. acidi lactici is briefly defined by Lehmann and Neumann (1904), and the only character which distinguishes it from B. coli is its constant non-motility. As they considered it to be identical with B. lactis aerogenes, it is difficult to assess the value of their findings.

TABLE 2.

Differential Characters of Coliform Types (1885-1904).

	motil^{y}	morph^{y}	colony	gel^{n} liqu^{n}	sucrose	starch	inulin	indole	gas ratio	VP
B. coli	+	thin		-	±	-	-	+	<1	-
B. lactis aerogenes	-	thick	whiter	-	(+)	(+)	(+)	$\bar{+}$	>1	+
B. cloacae	+			(+)	+			(+)		
B. pneumoniae (Fr)	-		viscid	-	+			-	<1	
B. acidi lactici	-			-				+		

($\bar{+}$ = more often negative than positive)

The chief distinguishing characters ascribed to the types recognised at this period are shown in Table 2.

Though it is not easy to rid one's mind of subsequent findings and to avoid laying emphasis on early observations which were later proved to be correct, it is possible to state fairly that amid all the apparent confusion a great deal had been truly discovered regarding the coliform bacteria as a group, the main types and their natural habitats.

It was universally agreed, for instance, that they were the predominant organisms in all kinds of faeces, and that B. coli (as defined in Table 2) was the commonest type therein. It was also fairly evident that the other types, especially B. lactis aerogenes, also occurred in faeces though in smaller numbers. The effect of food, disease, age and death had been studied and shown to make little difference to the faecal flora - B. coli remained the prevalent organism, with aerogenes holding second place. That similar conditions should exist in sewage, and contaminated water and soil was established, as was only to be expected, but the finding of coliform organisms (variously labelled "B. coli", etc.) in soils, waters and grains, whence the likelihood of faecal contamination had been carefully excluded, created a problem which could not then be solved, and caused considerable conflict of opinion. Sufficient attention was not paid during this period to the discovery of Voges and Proskauer (1898) and the observations of Russell and Bassett (1899), but this criticism can also be levelled at later work.

The high degree of interest shown during this time towards the coliform bacteria is not surprising when it is remembered that laboratory decisions regarding the potability of waters, for example, had to be made purely on the results of chemical analysis together with such bacteriological aid as was afforded by the total number of organisms cultivated per c.c. The inadequacy of such methods was beginning to be felt by hygienists whose minds became focussed on the possibility of using "B. coli" (in various senses) as an index of faecal contamination. Discussions raged as to the weight to be attached to the finding of "B. coli" (possibly only lactose-fermenters) in water, some authorities affirming that such was certain evidence of pollution, while others pointed to the fact that "B. coli" could be found almost everywhere including many sites apparently free from faecal associations and that, therefore, its presence in water was only to be expected. These latter denied any importance to the finding of "B. coli". It was only in 1900 (Pakes) that a quantitative approach was suggested and standards laid down which are remarkably in line with those of the present day.

Finally, from 1900 onwards it was becoming apparent that there were some differences between the coliforms most frequently found in faeces and those most often recovered from relatively clean soils and waters. The theory was, therefore, propounded that "typical B. coli" might suffer some degenerative changes when removed from its natural habitat. As shown already, not much evidence was produced to substantiate this hypothesis and all the experimental work done tended to disprove the occurrence of any fundamental changes, though it seemed possible that B. coli could lose its power to produce indole.

The Characters and Distribution of the Coliform Bacteria.

EARLY HISTORY AND LITERATURE

Period 1905 - 1910

With particular reference to the work of MacConkey (1905 to 1909)

Such was the state of affairs when MacConkey (1905) embarked on his monumental effort to make a comprehensive classification of the coliform group. (His only previous appearance in print was, as already mentioned, in a joint article with Hill (1901) on the use of bile-salt media.) He set off "with the object of ascertaining the distribution in nature of certain lactose-fermenting organisms which are by some grouped under the name Bacillus coli, but by others are regarded as belonging to a different class of organisms; a difference of opinion which is most probably the principal factor in causing the value of B. coli as an index of pollution to be such a vexed question among bacteriologists."

Having described the media and methods he had used in the experiments he was to recount, he turned to the literature and summarised the findings of previous workers concerning the various named types of coliform organisms.

B. coli

As regards B. coli he drew up a table of the earlier observations. This, in an abbreviated form, is reproduced in Table 3. His summing up is worth quoting:

"All these authors are agreed that the B. coli is usually a short bacillus with rounded ends; that it does not retain the dye when stained by Gram's method; that it is a non-sporing facultative anaerobe; that the growth on agar is a greyish-white layer; that the typical surface colony on gelatine is a "vine leaf", thin, filmy, translucent expansion; that gelatine is never liquefied; and that glucose is always fermented with the production of acid and gas. They differ, however, with regard to the

fermentation of the other carbohydrates and more particularly concerning cane-sugar. This is most probably due to the fact that organisms which give distinctly different fermentative reactions have been classed together as B. coli communis. But an organism can be termed B. coli communis (Escherich) only when it gives the constant reactions given by the original organism described by Escherich."

TABLE 3.

Some of the Reactions ascribed to B. coli.
(After MacConkey, 1905)

	Motility	Glucose	Lactose	Sucrose	Milk	Indole	$\frac{CO_2}{H_2}$	Gelatin liquefn.
Theobald Smith 1895	+	+	+	±	AC		½	
Grimbert 1896	+	+	+	-	AC	+		
Durham 1900-1	+	+	+	±	AC	±		
Ford 1901	+	+	±	+	AC	+		
Muir & Ritchie 1902	+	+	+		A±C	±		
Hewlett 1902	+	+	+		AC	+	½	
Houston 1903	±	+	+	±	AC	+		
Horrocks 1903	±	+	+	±	AC	+	<1	
Jordan 1903	+	+	+	±	AC	±	½	-
Eyre 1904	+	+	+	±	AC	+		
Savage 1905	±	+	+	±	AC	±		

B. lactis aerogenes (Escherich)

This species, he states, "might be described as a non-motile, Gram-negative, non-liquefying bacillus, a facultative anaerobe, producing acid and clotting milk, fermenting glucose, lactose and cane-sugar, and maybe also starch and inulin, and giving Voges and Proskauer's" reaction. He notes a distinction between aerogenes and B. pneumoniae (Friedländer) in that the latter ferments dulcite whereas the former does not.

B. cloacae (Jordan)

Of this organism he says: "The B. cloacae (Jordan) then might be described as an actively motile, Gram-negative, facultative anaerobic bacillus, fermenting glucose and cane-sugar, and maybe lactose, producing acid and clot in milk, and which almost invariably liquefies gelatine .."

B. pneumoniae (Friedländer)

His remarks here are: "It would appear that the B. pneumoniae (Friedländer) might be described as a non-motile, non-liquefying, Gram-negative, facultative anaerobic bacillus which produces acid in milk, with or

without clotting, and ferments glucose, lactose, and cane-sugar. In the blood of experimental animals it is frequently seen to have a capsule."

B. acidi lactici (Hüppe)

MacConkey makes no personal comments on this organism but merely summarises the accounts of previous workers.

From these findings he makes a useful tabulation which is reproduced in Table 4.

TABLE 4.

Reactions ascribed to five coliform types
(MacConkey, 1905)

	glucose	lactose	sucrose	milk	indole	motility
B. coli	+	+	±	AC	+	±
B. lactis aerog. (Esch)	+	+	+	AC	±	-
B. cloacae (Jordan)	+	±	+	A ± C	±	+
B. pneumoniæ (Friedländer)	+	+	+	A ±C	±	-
B. acidi lactici (Hüppe)	+	+	-	AC	+	-

He continues:

"It is obvious that, if reliance is placed solely upon the reactions given above, these organisms may be said to be closely related; and it is not surprising that by some the B. acidi lactici (Hüppe) and B. lactis aerogenes (Escherich) should be considered identical and practically non-motile forms of the B. coli, or that by others the B. lactis aerogenes is taken to be the same organism as the B. pneumoniae (Friedländer). Theobald Smith differentiates these bacilli by the amount of gas produced and by the "Gas Ratio", and his lead is followed by most of the American bacteriologists. But these distinguishing points are not universally accepted as of value.

"With the idea of, if possible, finding further points of difference I have tested the action of these and certain other organisms upon 14 carbohydrates and alcohols; have measured the "gas-ratio", and have noted the appearance of Voges and Proskauer's reaction." For this purpose he obtained sub-cultures of the classical

strains. He found that all the lactose-fermenters constantly fermented (with the production of acid and gas) the following substances: glucose, laevulose, galactose, maltose, arabinose, raffinose, mannite, sorbite and dextrin. (The paracolon types showed similar results - so far as they were tested - except for non-lactose-fermentation, and B. proteus vulgaris acted in the same way as the paracolons except for failure to attack sorbite). The remaining fermentable substances, starch, inulin, sucrose and dulcite, showed variable results as can be seen in Table 5 which is abridged from that of MacConkey.

TABLE 5.

Cultural Reactions of Certain Groups of Organisms.
(Abridged from MacConkey, 1905)

	lactose	sorbite	starch	inulin	sucrose	dulcite	VP	$\frac{CO_2}{H_2}$
B. coli communis (Escherich)	+	+	-	-	-	+	-	<1
B. acidi lactici (Hüppe)	+	+	-	-	-	-	-	<1
B. neapolitanus (Emmerich)	+	+			+	+	-	<1
B. pneumoniae (Friedlander)	+	+	-	-	+	+	-	<1
B. lactis aerog. (Escherich)	+	+	±	-	+	-	+	≧1
B. capsulatus (Heiffer)	+	+	±	-	+	-	+	≧1
B. cloacae (Jordan)	+	+	-	-	+	-	+	>1
B. paracolon (Day)	-	A			-	A	-	<1
B. paracdon (Le Sage)	-	+	-	-	-	+	-	<1
B. proteus vulgaris		-			+	-		

A = acid only.
< = less than. > = greater than.
≧ = greater than or equal to.

MacConkey continues:

"Now if we for the present neglect the question of motility (and indole which is not recorded and presumably not investigated - unfortunately -RDG) and restrict our consideration to the lactose fermenting organisms solely, a glance at the table shows that the differentiating points have reference to:-

(1) the fermentation, or otherwise, of cane-sugar and dulcite,
(2) the gas ratio,
(3) the appearance, or non-appearance of the ... reaction of Voges and Proskauer.

"And so, <u>just for the sake of classification in these experiments, the organisms have been divided</u>

(The underlining is mine - RDG)

arbitrarily into four groups according as they do, or do not, ferment cane-sugar and dulcite:"

GROUP	CANE-SUGAR	DULCITE
1.	-	-
2.	-	+
3.	+	+
4.	+	-

"Without implying that all the organisms in Group 1. are identical, one may speak of them as conforming in fermentative properties to the type of B. acidi lactici (Hüppe). Similarly, the B. coli communis (Escherich) may be taken as the fermentative type of Group 2, and the B. neapolitanus or B. pneumoniae (Friedländer) as representing Group 3. As regards Group 4 it will be seen from the results given below (referring now to his experiments still to be described) that this group may be subdivided into:

"Sub-group 1.	? B. coli	No liquefaction of gelatin; absence of VP reaction.
Sub-group 2.	B. lactis aerogenes	No liquefaction of gelatin; presence of VP reaction.
Sub-group 3.	B. cloacae	Liquefaction of gelatin ..; presence of VP reaction.
Sub-group 4.	Bacillus ?	Liquefying gelatin and producing a yellow pigment."

Except for the description of the Pasteur candle experiment (to which reference has already been made in the preceding section) the rest of this article deals with the isolation and examination of coliform bacteria from various sources. MacConkey commenced each experiment by emulsifying a portion of the sample in a tube of bile-salt broth, from which, after 24 or 48 hours' incubation at 37°C., plates were made using gelatin and agar. The plates were then incubated at 37°C. for 24 or 48 hours, when approximately 10 colonies were taken therefrom and "worked through the following media: nutrient broth, nutrient agar, nutrient gelatine, litmus milk, and bile-salt broths containing respectively glucose, lactose, mannite, cane-sugar, and dulcite. The cultures were also tested as regards their behaviour towards Gram's method of staining. At first potato was also used and media containing glycerine, starch and inulin, but it was soon found that these media had no great value in these experiments and so their use was discontinued."

"All the organisms isolated" he mentions later "had, unless otherwise stated, the following characters in common:-

"They were non-sporing, Gram-negative, facultative anaerobic bacilli, gave grey-white growths on agar and gelatine, without liquefaction of the latter; produced acid and clotting in milk; general turbidity, with formation of indole, in broth; and fermented glucose, lactose and mannite with the production of acid and gas. Their action on cane-sugar and dulcite is mentioned in connection with each experiment."

It should be, perhaps, stressed that his purpose became two-fold in the course of his experiments. His original object of investigating "the relative frequency of occurrence of certain lactose-fermenting bacilli" became almost subservient to a definite search for B. lactis aerogenes, for he was anxious to supply cultures of this strain to his colleague, Harden, who (1905) was studying its chemistry. His procedure in all but the last experiment (of which details are appended below), was to isolate 10 organisms per plate; test them for common characters, as above; segregate them (using sucrose and dulcite) into his 4 "Groups"; note the numbers, and then concentrate attention on the organisms in his Group 4. These he subjected to further tests as possible B. lactis aerogenes. As he repeatedly mentions that the Voges-Proskauer test was not used in these experiments, it must be presumed that he used for criteria in this respect such characters (besides non-liquefaction of gelatin) as non-motility, high gas volume and high gas (CO_2/H_2) ratio.

His experimental results may be summarised as follows:-

TABLE 6.

Source	Number of organisms in Group				Total	B. lactis aerog.	B. cloacae
	1	2	3	4			
Normal human faeces	15	21	0	0	36	0	0
Normal human faeces	33	17	16	12	78	4	0
Human faeces (diarrh. or enteric)	21	23	9	4	57	0	0
Normal hum. faec. (spec. diet)	14	32	11	13	70	0	0
Human faeces	83	93	36	29	241	4	0
Horse faeces	14	15	20	2	51	0	0
Cow faeces	8	12	23	5	48	0	1
Rabbit faeces (c typh. toxin)	2	8	1	19	30	0	0
Cat faeces (special diet)	15	58	31	36	140	0	0
Animal faeces	39	93	75	62	269	0	1
Milk	13	6	8	13	40	0	0
Human faeces & tap water	6	37	10	22	75	0	18
Total (all sources)	141	229	129	126	625	4	19

The numbers in Table 6 are transformed into percentages in Table 7.

TABLE 7.

Source	Percentage of organisms in Group				Total No. of orgms.
	1	2	3	4	
Normal human faeces	41	59	0	0	36
Normal human faeces	42	22	21	15	78
Human faeces (diarrh.)	37	40	16	7	57
Human faeces (special diet)	20	46	16	18	70
Human faeces *	34	39	15	12	241
Horse faeces	28	29	39	4	51
Cow faeces	17	25	48	10	48
Rabbit faeces (toxin)*	7	27	3	63	30
Cat faeces (special diet)*	11	41	22	26	140
Animal faeces	14	35	28	23	269
Human & Animal faeces *	24	36	22	18	510
Milk*	32½	15	20	32½	40
Human faeces & tap water	8	50	13	29	75
All sources	22·6	36·6	20·6	20·2	625

Though MacConkey quotes some of these percentages (marked with an asterisk in Table 7) and draws comparisons between certain sets of figures (e.g., those from man and cat on special diets), he draws no conclusions but contents himself with reporting the facts. It is evident that he had failed to establish any correlation between "Group" and source.

His last experiment arose out of his dissatisfaction with the extremely small number of B. lactis aerogenes he had recovered from faeces. Unable to reconcile his almost complete failure with the current opinion that this organism was commonly found in faeces, he added some human faeces to a flask of sterile tap-water (which was thereafter kept in a dark room), and inoculated samples (1 c.c.) therefrom at weekly intervals for 6 weeks, hoping thereby to give aerogenes a chance to multiply at the expense of coli and so come more into evidence. But once again he met with failure for no aerogenes bacilli were recovered.

In this experiment he recorded the indole-reaction of all but 10 of the organisms isolated, and applied to the 22 "Group 4" strains the following additional tests: motility, gas volume, gas ratio, Voges-Proskauer reaction, liquefaction of gelatin, and fermentation of starch and inulin. All 22 failed to ferment starch and inulin. 18

were motile, Voges-Proskauer-positive gelatin-liquefiers which he decided were B. cloacae (12 indole-negative and 6 indole-positive). The other 4 were non-motile, Voges-Proskauer-negative non-liquefiers (3 indole-negative and 1 indole-positive). Of the 53 organisms from Groups 1, 2 and 3, 43 were tested for indole-production, 23 being positive and 20 negative.

Though he once again failed to recover aerogenes, he had obtained evidence of something to which he does not draw attention: the much greater frequency with which indole-negative coliforms are obtained from water as compared with faeces; for, as no mention is made of indole results in the other experiments, we must understand from his introductory remarks that all 550 organisms were indole-positive, whereas of the 75 isolated in the tap-water experiment at least 35 were indole-negative.

Having omitted the Voges-Proskauer test from all but this last experiment, he devotes the final section of his article to a study of the test and applies it to a large number of organisms in various combinations. He notes that a positive reaction was obtained only when organisms of the B. lactis aerogenes or B. cloacae types were present.

Though these early investigations had mainly negative results, the article discloses evidence of much careful study. In particular is noteworthy the original description of the characteristic colonies on neutral-red bile-salt lactose agar of B. cloacae: "the surface colonies of B. cloacae, after about 20 hours in the incubator at 37°C., are usually found raised and opalescent, and some may have a red centre. With lapse of time they tend to run together into masses of mucoid-looking material with spots of deep red in them. The centres of all those colonies which remain discrete become red inside 48 hours. In 3 days the red colour may have entirely disappeared, being succeeded by a brownish-yellow colour."

Harden (1905) was meanwhile studying the chemical action on glucose of 56 coliform strains obtained from MacConkey. He investigated these together with laboratory cultures of B. coli communis (Escherich), B. acidi lactici (Hüppe), B. lactis aerogenes (Escherich), and B. cloacae (Jordan). Having grown each organism in an atmosphere of nitrogen in 500 c.c. of glucose-peptone-water, with 5 gm. of chalk, for a fortnight at 37°C., he then filtered off the insoluble residue and analysed the filtrate. He noted that, among other substances, alcohol and acetic acid were always produced, most often in approximately equal molecular proportions. In certain cases, however, the molecular ratio was more than 2·5 of

alcohol to 1 of acetic acid. The organisms which gave this latter ratio were B. lactis aerogenes (Escherich), B. cloacae (Jordan), and the strains from Subgroups 2 and 3 of MacConkey's Group 4, i.e., Voges-Proskauer-positive organisms. Harden concluded that "B. lactis aerogenes acts upon glucose in a totally different manner from B. coli communis and is therefore to be regarded as a distinct organism." Though the mechanism of the Voges-Proskauer reaction remained unexplained, it had, nevertheless, become associated with a fundamentally different bacterial metabolism.

Harden (1905-1906) persisted in his enquiry and succeeded (Harden and Walpole, 1906) in elucidating the chemical mechanism of the reaction by still closer study of the fermentation of glucose by B. coli and B. lactis aerogenes. Both organisms were proved to yield lactic, acetic, succinic and formic acids, together with ethyl alcohol, carbon dioxide and hydrogen; but, whereas in the case of B. coli these products quantitatively accounted for all the glucose fermented, in the case of aerogenes only two-thirds of the glucose fermented could be explained in this way. The remainder of the glucose was finally shown to be transformed into a substance (2:3 butylene glycol) which on oxidation yields acetyl methyl carbinol, and this in the presence of alkali and air is readily oxidised to diacetyl which produces a pink colour in the presence of peptone. In other words, organisms which give a positive Voges-Proskauer reaction do so by virtue of their ability to produce acetyl methyl carbinol, and B. coli lacks this power.

The inconveniently slow action on gelatin of the coliform liquefiers was the subject of MacConkey's next article (1906). He noted that B. cloacae, for instance, might show no change in the gelatin medium for 30 to 40 days, and that for practical purposes, as in the examination of water, such a delay would render the report valueless. "Besides," he continues, "the differentiation of B. cloacae from B. lactis aerogenes hinges upon the former's power of movement and of liquefying gelatin. It seemed, therefore, important to endeavour to devise some means by which it could be determined in a few days whether an organism belonged to the class of liquefiers." As the result of numerous experiments he showed that (1) by lowering the percentage of gelatin in the medium to 5%, (2) by using massive inoculations, and (3) by incubating at 37°C., the period necessary for demonstrating the liquefaction of gelatin by B. cloacae may be shortened to a week.

MacConkey next (1906a) turned his attention to the coliform organisms present in milk and obtained some noteworthy results. He collected altogether 24 samples

under varying conditions, e.g., direct from the cow with aseptic precautions, from the ordinary milking pail, from churns of mixed milk, and from country milk carts.

In the case of 11 samples, 10 of which were taken direct from the cow, and 1 from the milking pail, he found no gas-forming organisms present in 50 c.c. In 3 other samples (mixed milk) he found only chromogenic late-lactose-fermenters. From the remaining 10 samples (taken from clean milk in some cases and dirtier milks in others) he isolated 107 coliform bacteria, and subjected them to a large number of tests, including motility, indole-production, Voges-Proskauer reaction, fermentation of inulin, adonite, sucrose and dulcite, and liquefaction of gelatin. The results were reported for each organism.

Despite the inconclusive results of his 1905 experiments, he clung to the sucrose-dulcite classification and segregated the 107 organisms into the 4 groups, obtaining the following figures:-

	Organisms in Group:				Total No.
	1	2	3	4	
Number	9	42	35	21	107
Percentage	8·4	39·2	32·7	19·6	

He drew a parallel between these and the figures he had obtained in 1905 for cow faeces, noting that Group 2 and 3 organisms formed the majority. Then, arranging the milk samples according to their proximity to the source, he worked out further percentage figures and compared them with those obtained in 1905 which were concerned with town milk.

Samples	Percentage of organisms in Group:				Total number
	1	2	3	4	
Collected at farms	3·9	41·1	54·9	0	51
Collected from carts	12·5	37·5	12·5	37·5	56
Town milk	32·5	15	20	32·5	40

From this, he says, "it would seem that the farther we get from the source the greater the change in the Group proportions. Thus nearest the source we find Groups 2 and 3 taken together forming 96% of the organisms isolated. A short distance away they form 50%, and still farther away the number decreases to 35%. While Groups 1 and 4 increase from 3·9% at the source to 65% at the point farthest away."

In order to study the organisms individually and compare them with known strains, he published a list of named coliform bacteria whose reactions had been worked out. An abridged list is presented in Table 8.

TABLE 8.

Cultural Reactions of Classical Coliform Strains

(Abridged from MacConkey, 1906a)

	VP	Gel	Inulin	Indole	Adonite	Sucrose	Dulcite	Motility
B. grünthal	-	-	-	+	-	-	-	+
B. acidi lactici	-	-	-	+	+	-	-	-
B. coli communis	-	-	-	+	-	-	+	+
B. cavicida (Brieger)	-	-	-	+	-	-	+	+
B. neapolitanus	-	-	-	+	-	+	+	-
B. coscoroba	-	-	-	+	-	+	-	-
B. rhinoscleromatis	-	-	-	+	+	+	+	-
B. pneumoniae (Friedl.)	-	-	-	+	+	+	+	-
B. oxytocus perniciosus	+	-	+	-	+	+	+	-
B. lactis aerogenes	+	-	-	+	+	+	-	-
B. capsulatus (Pfeiffer)	+	-	-	+	+	+	-	-
B. cloacae (Jordan)	+	+	-	-	-	+	-	+
B. levans	+	+	+	-	-	-	-	+

It is of historical interest to note that he mentions (1) B. levans as having been described in 1894 by Wolffin as the cause of fermentation of dough, (2) B. coscoroba as having been cited in 1900 as the cause of an epidemic among swans, and (3) B. oxytocus perniciosus as isolated from stale milk in 1902. All the organisms in Table 8 had the common characters of coliform bacteria.

Using the reactions of these classical strains as differential criteria, he proceeded to try to identify each of the 107 organisms isolated. He showed that, by taking into account only the sucrose, dulcite, adonite, inulin, and Voges-Proskauer results together with the motility, some 64 different varieties were possible, but that only 14 were, in fact, obtained. Of these, he pointed out:

B. acidi lactici	was isolated		once,
B. coli communis	-	-	12 times,
B. neapolitanus	-	-	15 -
B. lactis aerogenes	-	-	twice,
B. cloacae	-	-	10 times,
B. oxytocus perniciosus	-	-	16 times,

while B. grünthal, B. pneumoniae (Friedländer) and B. coscoroba were not met with at all.

He quotes from the literature, summoning evidence to confirm his own conclusion that the presence of coliform organisms in milk is due to faecal contamination which takes place outside the udder.

At the end of his article he draws up a list of the tests usually employed, in the routine examination

of water and food-stuffs, to identify the coliform bacteria, i.e:

1. Morphology.
2. Motility.
3. Staining by Gram's method.
4. Character of the growth on nutrient gelatin.
5. Liquefaction of gelatin.
6. Action on milk.
7. Formation of indole.
8. Fermentation of glucose.
9. Fermentation of lactose.
10. Fermentation of sucrose.
11. Action on neutral-red.

All the organisms mentioned in Table 8 and the 107 coliforms isolated from milk, produced clotting in milk, fermented glucose, gave the neutral-red reaction (i.e., produced fluorescence), had much the same morphology, and the growths on agar and gelatin did not afford any assistance towards differentiating between them. He suggested, therefore, that tests 4,6,7,8, and 11 should be omitted and replaced by the following:

Fermentation of dulcite,
- - adonite,
- - inulin,
Voges-Proskauer reaction.

By so doing, he contends, we should get a finer differentiation without increase in work, and we should not be classing as B. coli organisms which may have little in common with, and have a distribution entirely different from that of, the B. coli communis.

In 1908, MacConkey published further details on the use of bile-salt media. He confirmed the superiority of neutral-red as indicator for differentiation on solid media, and described in detail his methods of preparation. He would not allow that colony characters on neutral-red bile-salt lactose media were of any value for distinguishing between the various lactose-fermenters, stating that the same strain might produce on the same plate colonies of widely different character and appearance. His mode of routine examination also received attention: primary inoculation of various amounts of, e.g., water into tubes of MacConkey lactose broth, followed, after 24-48 hours' incubation at 37°C., by subculture of tubes showing acid and gas on to MacConkey lactose agar. Red colonies developing thereon were picked off to agar slopes and thereafter put through the various differential tests. He notes that inosite is of value in this respect.

He made a number of enquiries into the inhibiting effect of varying strengths of bile-salt and showed that, with regard to the coliform, typhoid and allied organisms, his media permitted as free growth as any nutrient medium,

but greatly restricted the development of the air and soil bacteria. B. coli, he showed, could tolerate a higher concentration of bile-salt than the Voges-Proskauer-positive coliforms. The strength of bile-salt recommended for regular use was 0·5%.

While MacConkey's name is prominently associated with the coliform bacteria mainly by reason of the media he introduced for their primary isolation, his work on classification was also notable. His greatest contribution in this latter respect was his final paper (1909) in which, but for an error of interpretation, he was practically abreast of modern times, and certainly years ahead of his contemporaries.

He commenced (1909) by expressing dissatisfaction with the differing standards by which "B. coli" was defined, showing that those in England (according to Savage, 1906) and in America (according to Prescott and Winslow, 1908), while agreeing as regards morphology, the production of acid and clot in milk, the fermentation of glucose and lactose, and the non-liquefaction of gelatin, placed differing importance upon such attributes as motility, the formation of indole, and the reduction of nitrates. The divergence of opinion is indicated in Table 9.

TABLE 9.

Standardised Character of (typical) B. coli.

	English Standards (Savage, 1906)	American Standards (Prescott and Winslow, 1908)
Typical non-sporing rod	+	+
Motile	+	(+)
Gram-negative	+	+
Acid and clot in milk	+	+
Gelatin not liquefied	+	+
Ferment^{n} of glucose	+	+
Ferment^{n} of lactose	+	+
Gas (CO_2/H_2) less than 1	(+)	+
Formation of indole	(+)	+
Reduction of nitrates	(+)	+

\+ = obligatory attribute
(+) = non-obligatory attribute usually possessed

The differences are even greater than shown in the table, for Savage did not himself consider motility as an essential character of B. coli, but regarded its absence as of no significance. For these reasons, MacConkey enquired into the possible value of each of the tests in use at that time and dismissed the following as valueless for differential purposes:-

Morphology.	Too variable.
Growth in broth, blood-serum, and on potato.	Not characteristic.
Action on litmus milk.	Milk always clotted.
Production of acid from litmus whey.	Not worth the trouble.
Fluorescence in neutral-red.	Shared with many other organisms.
Reduction of nitrates.	Shared with many other organisms.
Growth on nutrient gelatin.	Not characteristic.

While some of the above might serve to distinguish the coliform group from other organisms, they had no internal differential value, and others were useless in both respects. He advocated the replacement of these tests by fermentation reactions (e.g., glucose, lactose, sucrose, dulcite, adonite and inulin), the Voges-Proskauer reaction, indole-production, and motility. This last was, he admitted, of doubtful value but was so easily tested that it was worth further trial. He had revised his opinion (1906a) regarding indole-production because of improved results obtained with a new reagent. In 1906 he had demonstrated the presence of indole by using sodium nitrite and sulphuric acid, and had noted considerable variation in his results. Böhme (1905) had however introduced Erlich's reagent for this test (paradimethylaminobenzaldehyde with absolute alcohol and hydrochloric acid) and MacConkey, who was employing this in 1908, now felt that the indole test constituted a reliable criterion. As regards fermentation tests, he disagreed with Savage (1906) who, after giving a definition of B. coli (see Table 9), stated: "Organisms with all the above characteristics, whether they ferment saccharose, dulcite, etc., or not, can all be spoken of as B. coli."

To confirm his views on the inadequacy of the tests usually employed, he applied them together with the additional ones he had recommended to some 500 lactose-fermenting organisms which he had isolated from various sources. Altogether 76 samples had been studied and these consisted of the following:

Human faeces	20	Soil	2	Bran	1
Horse faeces	11	Pond water	4	Old hay	1
Calf faeces	7	Rain water	1	Malt	1
Goat faeces	2	Roof washings	2	Baker's yeast	1
Goose faeces	6	Oats	2	Cheese	1
Pig faeces	1	Beans	1	Human sputum	9
Cesspool sewage	1	Ear of corn	1	Human pus	1

The organisms were recovered in the usual manner by primary incubation in bile-salt lactose broth and plating on MacConkey agar. Pure colonies were then subjected to the tests with the results given in Tables 10 and 11 which are reproduced almost in their entirety from those

TABLE 10.

Characters of the bacilli isolated (MacConkey, 1909)

No.	Name (if any)	Lactose	Litmus milk	Gelatin	Gram's stain	Motility	Indole	Reduction of nitrates	Sucrose	Dulcite	Adonite	Inulin	Inosite	VP reaction
1		+	+	-	-	+	+	+	-	-	+	-	-	-
2	B. acidi lactici (Hüppe)	+	+	-	-	-	+	+	-	-	+	-	-	-
3	B. levans	+	+	+	-	+	-	+	-	-	-	+	-	+
4	B. grünthal	+	+	-	-	+	+	+	-	-	-	-	-	-
5	B. vesiculosus	+	+	-	-	-	+	+	-	-	-	-	-	-
6		+	+	-	-	-	-		-	-	-	-		+
7		+	+	-	-	+	-	+	-	-	-	-	-	-
8	B. coli mutabilis (Massini)	+	+	-	-	-	-		-	-	-	-	-	
33		+	+	-	-	+	+		-	+	+	-	-	-
34	B. coli communis, B. cavicida	+	+	-	-	+	+	+	-	+	-	-	-	-
35	B. schafferi	+	+	-	-	-	+	+	-	+	-	-	-	-
36		+	+	+	-	+	-	+	-	+	-	-	-	-
65	B. oxytocus perniciosus	+	+	+	-	-	+	+	+	+	+	+	+	+
66		+	+	-	-	-	+	+	+	+	+	-	-	-
67		+	+	-	-	-	-	+	+	+	+	-	+	+
68	B. friedländer, B. rhinoscleroma	+	+	-	-	-	-	+	+	+	+	-	+	-
69		+	+	+	-	+	-	+	+	+	-	+	-	+
70		+	+	+	-	+	-		+	+	-	+	-	-
71	(B. coli communior)	+	+	-	-	+	+	+	+	+	-	-	-	-
72	B. neapolitanus	+	+	-	-	-	+	+	+	+	-	-	-	-
73		+	+	+	-	+	-	+	+	+	-	-	-	+
74		+	+	-	-	+	-		+	+	-	-		-
75		+	+	-	-	-	-		+	+	-	-	+	+
97		+	+	+	-	-	+	+	+	-	+	+	-	+
98		+	+	-	-	-	-	+	+	-	+	+	+	+
99		+	+	-	-	-	-	+	+	-	+	+	+	-
100		+	+	-	-	+	+		+	-	+	-		-
101		+	+	-	-	-	+	+	+	-	+	-	+	-
102		+	+	+	-	+	-		+	-	+	-		+
103	B. lactis aerogenes, B. capsulatus	+	+	-	-	-	-	+	+	-	+	-	+	+
104	B. gasoformans non-liquefaciens	+	+	-	-	-	-	+	+	-	+	-	A	±
105		+	+	+	-	+	-	+	+	-	-	+	-	+
106		+	+	-	-	+	+		+	-	-	-	-	-
107	B. coscoroba	+	+	-	-	-	+	+	+	-	-	-	-	-
108	B. cloacae	+	+	+	-	+	-	+	+	-	-	-	-	+
109		+	+	-	-	+	-		+	-	-	-	A	-

of MacConkey. It will be noticed that he retained his sucrose-dulcite groups. To explain the numbers used in the first column he writes: "If we add, as further tests (to the fermentation of sucrose and dulcite), the action on adonite and inulin, the presence or absence of motility, of indole, and of the Voges-Proskauer reaction, it is possible to form 128 combinations, or, to put it in other words, we might isolate 128 varieties of lactose-fermenting

TABLE 11.

Distribution of the bacilli isolated (MacConkey, 1909)

No.	Name	Human faeces	Horse faeces	Calf faeces	Goat faeces	Pig faeces	Goose faeces	Cesspool sewage	Soil	Pond water	Rain water	Roof washings	Oats	Beans	Bran	Cheese	Baker's yeast	Malt	Ear of corn	Human pus	Human sputum	Totals
1		9	3	2	2	-	-	-	-	-	-	-	-	-	-	5	-	-	-	-	-	21
2.	B. acidi lactici	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
3.	B. levans	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
4.	B. grünthal	8	1	-	-	-	5	4	-	-	-	-	-	-	-	-	-	-	-	-	-	18
5.	B. vesiculosus	33	-	-	-	3	1	-	1	-	-	-	-	-	-	3	-	-	1	5	1	48
6.		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	1
7.		1	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	2
8.	B. coli mutabilis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
33.		-	5	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	9
34.	B. coli communis	37	15	3	13	1	3	1	-	-	2†	-	4	-	-	-	-	-	-	-	3	82
35.	B. schafferi	11	1	-	-	3	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	16
36.		1	1×	-	-	-	-	-	-	-	-	-	-	-	-	-	3	3×	-	-	-	8
65.	B. oxytocus perniciosus	1	-	-	-	-	-	-	4	-	-	-	-	-	-	3	-	-	-	-	-	8
66.		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4	4
67.		1	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3
68.	B. friedländer	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
69.		-	3×	-	-	-	-	-	-	-	-	6×	1	-	-	-	-	-	-	-	-	10
70.		-	-	-	-	-	-	-	-	-	1×	-	-	-	3	-	-	-	-	-	-	4
71.	(B. coli communior)	42	32	30	-	-	9	-	6	3	1	5	1	-	-	-	4	-	5	5	-	143
72.	B. neapolitanus	15	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	17
73.		-	1×	-	-	-	-	-	-	1×	-	7×	1×	3×	-	-	-	6×	5×	-	-	24
74.		1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
75.		-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	1
97.		1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
98.		-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	1
99.		-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	1
100.		1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
101.		1	-	-	-	-	-	-	-	-	-	4	-	-	-	-	-	-	-	-	-	5
102.		-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	2
103.	B. lactis aerogenes	8	-	-	-	-	-	-	-	-	-	-	-	-	-	5	-	-	-	-	2	15
104.	B. gasoformans	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	2
105.		-	-	-	-	-	-	-	-	-	-	2	-	-	-	7	-	-	-	-	-	9
106.		2	1	-	1	-	4	-	-	-	-	-	-	-	-	-	-	-	-	-	1	9
107.	B. coscoroba	1	-	1	-	-	1	-	3	-	-	-	-	-	-	-	-	-	-	-	-	6
108.	B. cloacae	-	2	-	-	-	-	-	1	5	3	3	-	-	-	-	-	-	-	-	-	14
109.		2	-	-	-	-	-	-	-	-	-	2	-	-	-	2	3	-	-	-	-	9
	Totals	178	67	40	17	7	23	6	16	11	7	31	9	3	3	26	10	9	11	10	13	497

×yellow liquefier.
†yellowish.

bacilli. But only about one quarter of this number have been met with, and so in order to allow for the correct placing of the other bacilli, 32 numbers have been assigned to each group."

A study of Table 10 shows that all the bacilli fermented lactose, clotted milk, and were Gram-negative; that in all cases which had been tested nitrates were reduced; that the majority did not liquefy gelatin, and those which did so liquefied so slowly as to be classed in routine work as non-liquefiers; that motility and the indole tests were positive in some cases and negative in others. The morphology was not noted as it had proved to be of no differential value.

"Now," says MacConkey, "according to the tests usually employed, all these bacilli would be classed as B. coli. But if we kept them long enough we should find that in several cases gelatin was liquefied and we should be in the position of having classified a liquefying organism as B. coli, though all bacteriologists are agreed that B. coli is not a liquefying organism.

"It has been suggested that the error from this cause is so small that it may be neglected, but an enumeration of the liquefiers in Table II (i.e. 11) shows that the error would be more than 10% and therefore cannot be ignored. It follows that the present methods of differentiation are not adequate and that a change is necessary."

The change he recommends is the introduction of dulcite, adonite, and inulin fermentation, and the Voges-Proskauer reaction, together with the omission in future of colony characters on gelatin, and the action on milk, glucose and neutral red.

His faith in the Voges-Proskauer test, which had gradually increased since 1905, became weakened in 1907 when he isolated some organisms which gave variable and doubtful results, but he still considered it to be of value. By using his massive inoculation method he proved that B. oxytocus perniciosus was really a liquefier (cf. Table 8), and notes that his results with B. cloacae (1906) had been confirmed by Ferreira, Horta and Paredes (1908).

"Coming now to Table II (i.e. 11)," he continues, "we are struck by the distribution of the bacilli isolated. If all these organisms were the same bacillus we might reasonably expect that they should be distributed fairly evenly through out the samples. But such is not the case.

"The B. levans, for instance, is so rare that it has not been met with once in 497 bacilli, and yet B.

levans has been stated to be identical with B. coli (Papasotiriu, 1902).

"On the other hand, 7 of the varieties given in Table I (10) claim 87%, and 3 varieties 62%, of the 178 bacilli obtained from human faeces, and of the 154 organisms with an origin in animal faeces 68% belong to 2 varieties and 46% to a single variety. If we consider the 497 bacilli as a whole we find that 54·9% of them belong to one of other of 3 varieties. If all these bacilli are B. coli we must allow that certain varieties of this organism are very common in faeces and material exposed to faecal contamination, while certain other varieties are rare in faeces and more common in other materials.

"This suggests that the term B. coli, as at present used, is not a happy one; it is too comprehensive, and it would be better to avoid using it. There can be little doubt that all these bacilli have already been isolated, and partially described under some name, but I have found it impossible to obtain more than a few named cultures. For the others we must be content to use numbers until we find out the names by which they should properly be known.

"I think I have now brought forward sufficient evidence to justify me in once more urging the adoption in routine work of the series of tests recommended in this paper."

He concludes the article with some notes upon the reliability of fermentation reactions in the coliform group, since by some workers these reactions had been stated to be too inconstant for classification purposes. He cites his own experience to the contrary and refers to Savage (1906) and others. "Nor must it be forgotten," he effectively adds, "that this very kind of reaction is considered of great value in the differentiation of B. coli, B. typhosus and B. enteritidis (Gaertner). If they are looked upon as reliable enough for this purpose why should they not be equally of value in separating B. coli from B. lactis aerogenes and similar organisms?"

As the result of prolonged trials with a great many fermentable substances, he came to the conclusion that "for the purpose of differentiating the lactose-fermenters we can be content to use lactose, saccharose, dulcite, adonite, inulin, inosite and maybe mannite."

His conclusion reads:

"It has been shown that the tests at present in general use do not allow us to differentiate adequately the lactose-fermenting bacilli from each other. It has also been shown that by the substitution and addition of

certain other tests we shall gain in accuracy with little increase in labour, and that we shall thus have a fair prospect of being able to pick out those organisms which are most closely associated with faeces and put the bacteriological examination of water supplies upon a firmer basis than that upon which it stands at present."

The additional information which can be deduced from this investigation will be indicated in the discussion to follow presently; but the work of contemporary bacteriologists deserves some mention.

Reference has already been made to Böhme (1905) who, by introducing the use of Erlich's reagent for the indole test, effectively increased the accuracy of this reaction. The Erlich reagent (benzaldehyde-alcohol-HCl mixture) was first added, and then, if no colour were obtained, a saturated solution of potassium persulphate could be added as a reinforcing agent.

Though Longley and Baton (1907) considered that the gas volume and gas ratio were unreliable, and were the cause of MacConkey's final omission of these tests, interest was revived therein after Keyes (1909) evolved a more accurate method for estimating the volumes of the gases produced. The sequel to this belongs to the next section.

MacConkey's example was the basis for a still more elaborate classification which was introduced by Bergey and Deehan (1908). They used the same tests as MacConkey (motility, indole, V-P, gelatin, sucrose, dulcite, adonite and inulin) and formed a separate sub-group for each of the 256 possible combinations.

Winslow and Walker (1907) conducted similar researches to those of MacConkey; from human faeces they obtained 52 glucose-fermenting strains of which 31 were typical B. coli (as regards milk, nitrates, indole and gelatin). They tested 25 of these on sugars and only one proved to be sucrose-positive and dulcite-negative. They recovered no glucose-fermenting organisms from 40 samples of grass, but from 178 samples of grains they isolated 50 such strains of which only 3 gave acid colonies on litmus lactose agar and all 3 were gelatin-liquefiers. They called attention to the close correlation between fermentation of sucrose and that of raffinose.

Ferreira, Horta and Paredes (1908) isolated 117 coliform bacilli from human faeces. Only 8 of these gave a positive Voges-Proskauer reaction. All were non-liquefiers and only one failed to produce indole.

Rivas (1908) considered that many organisms said to belong to the colon group had no connection with B. coli. Like MacConkey, he wanted to narrow the conception of this bacillus as applied to the organisms present in

milk, water, etc. He also suggested some new tests but these were not generally adopted for routine purposes.

Summary and Discussion
(1905-1910)

As has been described, MacConkey was in 1905 acutely conscious of the unsatisfactory divergence of opinion as to what constituted the exact characters of "B. coli", and it has been noted that, in an effort to obtain a better definition of the coliform types, he collected as many classical coliform strains as possible and subjected them to a large number of tests. All but 4 of these tests appeared useless for providing distinguishing features between the strains investigated. The 4 helpful tests were:

The Voges-Proskauer reaction.
The gas-ratio.
Fermentation of sucrose.
Fermentation of dulcite.

In choosing the last two (in preference to the others) for the purposes of classification, MacConkey made his greatest mistake, and laid a trap into which many later workers fell. It is difficult to understand how he failed to notice the absolute correlation between the Voges-Proskauer test and the gas-ratio (see Table 5) but it must be remembered that he was new to the Voges-Proskauer test and took some years before placing much reliance upon it. Having chosen quite "arbitrarily", as he says, the sucrose-dulcite basis for distinguishing between the classical strains, he applied it to the organisms he isolated in a perfectly legitimate manner. It is for the purpose of emphasising this that his remarks on the subject have been quoted in full. Each of his first three groups was merely <u>represented</u> by a classical strain. Unfortunately, later writers have misunderstood this and have identified the sucrose-dulcite result with the representative name, i.e. they have assumed that all coliform organisms which are sucrose-negative and dulcite-positive must be B. coli communis, for example. Even such an eminent authority as the M.R.C. System of Bacteriology misquotes MacConkey in this respect.

At the risk of labouring the point, I beg to submit an example which may indicate the absurdities of the above position. If I set out to investigate the population of the town in which I live, and, instead of choosing the obvious differential criteria of sex and age, I preferred (for reasons of my own) to count the numbers of people who had fair and dark hair, and blue and brown eyes, respectively, I might obtain some interesting results, though I should of course finish up without any

information as to the number of men, women and children. In the course of my enquiries I might, perhaps, note that a prominent garage proprietor belonged to the first "Group" - with fair hair and blue eyes; that fair hair (possibly with dark roots) together with brown eyes were commoner among the women, viz. Mrs. B. the hairdresser; that commonest of all were dark hair and brown eyes, as notably presented by Doctor C.; and that dark hair and blue eyes though relatively uncommon were quite often seen in babies. Tabulating, like MacConkey, one would have:

Group	Hair	Eyes	e.g.
1	Fair	Blue	Mr. A. the garage-owner
2	Fair	Brown	Mrs. B., hairdresser
3	Dark	Brown	Dr. C.
4	Dark	Blue	Baby D.

One could quote the percentages of each group which one found in the town and compare them with those found in the more rural districts, and one could possibly come to some conclusions as to the relative frequency of such combinations in town and country. But what one could not assume is that everyone with fair hair and blue eyes must be a garage-proprietor, or that only those with dark hair and brown eyes can hope to become doctors. This is, nevertheless, precisely the kind of assumption that subsequent workers made of MacConkey's proposed classification.

MacConkey, himself, did likewise to a certain extent, e.g., because the strain B. lactis aerogenes (Escherich) was sucrose-positive, dulcite-negative he assumed that all similar types must behave similarly. Thus he looked for Voges-Proskauer-positive coliforms only among his fourth group, and then expressed surprise and disappointment at finding so few. A glance at his later work (especially Table 10) will show how many possible chances he neglected. It is, indeed, most unfortunate that, to begin with at least, the two most fundamental tests then available (Voges-Proskauer reaction and gelatin-liquefaction) were reserved for subdividing his fourth group.

It is not, therefore, surprising that the percentages of his "Groups" failed to show any appreciable correlation with environment. Each group was a veritable conglomeration.

The value of the Voges-Proskauer test for distinguishing the aerogenes and cloacae types was, however, becoming more generally appreciated, and with the advent of Harden's explanation of the chemistry of the reaction came the realisation that the test was of considerable importance. MacConkey invariably included it from 1906 onwards though he never put it where it belonged - in the first place.

In his article on milk (1906) he applied the Voges-Proskauer test to every coliform bacillus he isolated, and reported all his results for each individual organism. Though, for the first time, he managed to draw tentative conclusions regarding his group-proportions here, it is more important that his work admits of closer scrutiny than the earlier article where the Voges-Proskauer test was not employed and the other results not quoted. It had already been generally agreed that, while certain coliform bacteria could liquefy gelatin, B. coli could neither do this nor give a positive Voges-Proskauer reaction. It is possible, on this basis, to subdivide the 107 organisms which MacConkey recovered from milk, as shown in Table 12.

TABLE 12.

Coliform bacteria obtained by MacConkey from milk

(Adapted from MacConkey, 1906)

VP	Gel	Possible type	No.	Remarks
+	+	(cloacae)	10	all indole-neg.
+	-	(aerogenes)	21	15 ind_+, 6 ind_-
-	-	(coli)	76	70 ind_+, 6 ind_-

Though purists like MacConkey would not have allowed the names suggested above, anyone, even in 1906, would have agreed that at least 31 of the 107 bacteria were certainly not B. coli, whereas at least 70 were possible B. coli types. On the same basis it is possible to investigate the numbers present in the various milk samples.

TABLE 13.

Coliform Bacteria obtained by MacConkey from milk.

(Adapted from MacConkey, 1906)

Samples	Coliform organisms giving reactions:			
	VP_+Gel_-	VP_+Gel_+	VP_-Gel_- indole$_-$	VP_-Gel_- Indole$_+$
Collected at farms	13	0	0	38
Collected from carts	8	10	6	32

Though the numbers are rather small to support more than tentative conclusions, it is plain that B. coli types form the majority in each kind of sample. This is, of course, what one would expect as the contamination has been proved to be faecal in nature. As regards the aerogenes-cloacae types there is not much to choose - 13 in the fresher samples as against 18 in the others - but even on this basis the trend is the same as that found by MacConkey, though the degree of difference is less. By neither one method or the other, however, can anything very conclusive be proved.

TABLE 14.

Characters of the bacilli isolated by MacConkey

(Adapted from MacConkey, 1909)

No.	Name (if any)	VP reaction	Gelatin	Inulin	Imosite	Indole	Adonite	Sucrose	Dulcite	Motility	Modern types
3	B. levans	+	+	+	-	-	-	-	-	+	
69		+	+	+	-	-	-	+	+	+	
73		+	+	-	-	-	-	+	+	+	
102		+	+	-		-	+	+	-	+	Cloacae
105		+	+	+	-	-	-	+	-	+	
108	B. cloacae	+	+	-	-	-	-	+	-	+	
65	B. oxytocus perniciosus	+	+	+	+	+	+	+	+	-	
97		+	+	+	-	+	+	+	-	-	Oxytocus
6		+	-	-		+	-	-	-	-	Aerogenes, type II.
67		+	-	-	+	-	+	+	+	-	
75		+	-	-	+	-	-	+	+	-	
98		+	-	+	+	-	+	+	-	-	Aerogenes, type I.
103	B. lactis aerogenes	+	-	-	+	-	+	+	-	-	
104	B. gasoformans	+	-	-	A	-	+	+	-	-	
36		-	+	-	-	-	-	-	+	+	
70		-	+	+	-	-	-	+	+	+	Intermediate, type I.
99		-	-	+	+	-	+	+	-	-	
68	B. pneumoniae (Fried)	-	-	-	+	-	+	+	+	-	Intermediate, type I.
101		-	-	-	+	+	+	+	-	-	Intermediate, type II
109		-	-	-	A	-	-	+	-	+	
74		-	-	-		-	-	+	+	+	
7		-	-	-	-	-	-	-	-	+	Coli, type II
8	B. coli mutabilis	-	-	-	-	-	-	-	-	-	
1		-	-	-	-	+	+	-	-	+	
2	B. acidi lactici	-	-	-	-	+	+	-	-	-	
4	B. grunthal	-	-	-	-	+	-	-	-	+	
5	B. vesiculosus	-	-	-	-	+	-	-	-	-	
33		-	-	-	-	+	+	-	+	+	
34	B. coli communis	-	-	-	-	+	-	-	+	+	Coli, type I
35	B. schafferi	-	-	-	-	+	-	-	+	-	
66		-	-	-	-	+	+	+	+	-	
71	(B. coli communior)	-	-	-	-	+	-	+	+	+	
72	B. neapolitanus	-	-	-	-	+	-	+	+	-	
106		-	-	-	-	+	-	+	-	+	
107	B. coscoroba	-	-	-	-	+	-	+	-	-	
100		-	-	-		+	+	+	-	+	

TABLE 15.

Distribution of the bacilli isolated by MacConkey

(Adapted from MacConkey, 1909)

No.	Name (if any)	Human faeces	Animal faeces	Cesspool sewage	Water	Soil	Cereals & foods	Sputum & pus	All sources	Modern types
3	B. levans	-	-	-	-	-	-	-	0	Cloacae
69		-	3	-	6	-	1	-	10	
73		-	1	-	8	-	15	-	24	
102		-	-	-	2	-	-	-	2	
105		-	-	-	2	-	7	-	9	
108	B. cloacae	-	2	-	11	1	-	-	14	
65	B. oxytocus perniciosus	1	-	-	-	4	3	-	8	Oxytocus
97		1	-	-	-	-	-	-	1	
6		-	-	-	-	-	-	1	1	Aerogenes II
67		1	2	-	-	-	-	-	3	Aerogenes I
75		-	-	1	-	-	-	-	1	
98		-	-	-	-	-	1	-	1	
103	B. lactis aerogenes	8	-	-	-	-	5	2	15	
104	B. gasoformans	-	-	-	2	-	-	-	2	
36		1	1	-	-	-	6	-	8	Intermediate I
70		-	-	-	1	-	3	-	4	
99		-	-	-	-	-	1	-	1	
68	B. pneumoniae (Fried.)	1	-	-	-	-	-	-	1	
101		1	-	-	4	-	-	-	5	Intermediate II
109		2	-	-	2	-	5	-	9	Coli II
74		1	-	-	-	-	-	-	1	
7		1	-	-	-	1	-	-	2	
8	B. coli mutabilis	-	-	-	-	-	-	-	0	
1		9	7	-	-	-	5	-	21	Coli I
2	B. acidi lactici	1	-	-	-	-	-	-	1	
4	B. grunthal	8	6	4	-	-	-	-	18	
5	B. vesiculosus	33	4	-	-	1	4	6	48	
33		-	9	-	-	-	-	-	9	
34	B. coli communis	37	35	1	2	-	4	3	82	
35	B. schafferi	11	4	-	-	-	1	-	16	
66		-	-	-	-	-	-	4	4	
71	(B. coli communior)	42	71	-	9	6	10	5	143	
72	B. neapolitanus	15	1	-	-	-	-	1	17	
106		2	6	-	-	-	-	1	9	
107	B. coscoroba	1	2	-	-	3	-	-	6	
100		1	-	-	-	-	-	-	1	
	Totals	178	154	6	49	16	71	23	497	

In his 1909 work, on the other hand, where the number of organisms isolated was much greater (497), the sources more varied, and the results at least equally fully recorded, it can be shown that only his adherence to the sucrose-dulcite grouping prevented MacConkey from achieving an up-to-date correlation of type with source. As has been suggested above, a coliform organism which liquefies gelatin or gives a positive Voges-Proskauer reaction cannot be called B. coli. Similarly, if we insist on the capacity to form indole as an obligatory property of "typical B. coli" we can exclude from this category MacConkey's strains Nos. 7, 8, 68, 74, 99 and 109. The various strains have been segregated on this basis in Table 14 where the only strain excluded from the true coli class in the light of later knowledge has been No. 101 - on account of its power to ferment inosite. The names suggested in the final column are based on the present Ministry of Health (1939) classification and Malcolm (1938) to which reference will later be made. Study of this table shows that for one reason or another only 13 (out of the 36 obtained by MacConkey) varieties are present which by modern standards cannot, on the tests used, be excluded from the "typical B. coli" category. By the standards which could be applied in 1909 only one further strain need have been included (No. 101) and as only 5 members of this variety were found the interpretation would have been materially the same.

If all the strains as now arranged are studied with reference to their relative frequency in the various samples (viz: Table 15) the concentration of the coli types in the faecal specimens becomes dramatically obvious. These coli types, for example, form 160 out of the 178 coliforms obtained from human faeces, 145 of the 154 from animal faeces, and 5 of the 6 from cesspool sewage. This preponderance does not, however, exist in the other sites, as may be seen more clearly in Table 16.

TABLE 16.

Summary of results given in Table 15

Source	No. of organisms belonging to		Total
	typical coli types	other types	
Human faeces	160	18	178
Animal faeces	145	9	154
Cesspool sewage	5	1	6
Water	11	38	49
Soil	10	6	16
Cereals & other foods	24	47	71

The relative frequency of the cloacae types in water and foods (29 and 23 respectively) is noteworthy.

The remarks quoted from his article show that MacConkey was aware that certain coliform varieties which were extremely common in faeces (and faecally contaminated material) were not so common elsewhere. Had he not placed undue emphasis on his sucrose-dulcite classification, he could have organised his results as effectively as has been done in Tables 14-16. He nevertheless deserves great credit, for he provided data which can be checked and confirmed with almost the same accuracy as can be obtained with the improved methods of to-day. In fact, it can be said that no better work has since been done as regards the definition of individual coliform strains, though much done on this subject has been considerably worse.

In his approach to the group as a whole, MacConkey also showed his genius. His thorough investigation of the value of each of the tests used, and his dismissal of the many superfluous ones, were of great benefit to later workers.

MacConkey is rightly regarded as the greatest of the early contributors to the bacteriology of the coliform group, and his main services may be summarised as follows:

(1) The invention of media which are still unsurpassed for routine investigation of the coliform group.

(2) The demonstration of the futility of many of the routine tests in common use, and the introduction of new useful differential tests with which to replace the former.

(3) The scrutiny of individual coliform strains, the definition of which is good by present-day standards, i.e., after the lapse of 35 years' intensive research.

(4) The elaboration of a method for demonstrating the liquefaction of gelatin in a reasonably rapid manner.

The only contemporary work which is worthy of notice was the discovery by Harden (1905-6) of the chemistry of the Voges-Proskauer reaction, and the original description by Eijkman (1904) of a high temperature fermentation test for B. coli. This latter was not not developed for some 30 years and will receive attention in a later context.

The Characters and Distribution of the Coliform Bacteria.

HISTORY AND LITERATURE

Period 1910 - 1920.

The work of MacConkey was most closely followed by Clemesha (1912) who studied in India the coliform flora of human and bovine faeces. He isolated a total of 2236 lactose-fermenters, and, using MacConkey's classification and tests, segregated all but 22 of his cultures into the 36 strains described by MacConkey. The 22 cultures, he found, resolved themselves into 4 new varieties, to which he gave the numbers 10, 38, 39 and 110 respectively. Like MacConkey, he investigated the frequency of each strain in the two sites and confirmed MacConkey's finding that Voges-Proskauer-positive organisms occurred in faeces only in comparatively small numbers. The results of both workers are summarised in Table 17.

TABLE 17.

The relative frequency in faeces of Voges-Proskauer-positive coliforms

Source	Reference	VP-		VP+		Total
		No.	%	No.	%	No.
Human faeces	MacConkey (1909)	167	93·8	11	6·2	178
	Clemesha (1912)	1143	94·7	64	5·3	1207
Animal faeces Cow faeces	MacConkey (1909)	146	94·8	8	5·2	154
	Clemesha (1912)	890	86·5	139	13·5	1029

The close similarity in the percentage figures for human faeces is particularly remarkable when it is considered that Clemesha's subjects were natives whose diet was almost entirely vegetarian. Clemesha noted the somewhat higher incidence of the aerogenes-cloacae types in cow dung, and observed that B. cloacae was extraordinarily common in certain of the specimens.

Clemesha (1912a) also turned his attention to waters in India, and showed that B. lactis aerogenes was rare in recently polluted water but became much more frequent some 5 to 15 days after the pollution. He noted that aerogenes was common in surface waters after rainy seasons, and that cloacae was the predominant organism after dry seasons. He studied the viability of the 3 coliform types in polluted streams and stored water, and found that while B. coli did not long survive therein aerogenes and cloacae had greater powers of resistance, especially the latter. He considered that in India multiplication of B. lactis aerogenes was a normal phase in the natural self-purification of waters. The experience of Houston (1911) in England had been exactly the opposite. Having examined 532 lactose-fermenting bacteria isolated from

water he noted that the Voges-Proskauer-positive types accounted for the following percentages of the total coliform organisms obtained:

from raw river water, 10·3%,
from stored river water, 5·3%,
from stored and filtered river water, 3·2%.

In his view, therefore, there was no evidence of increase in the Voges-Proskauer-positive types during storage.

MacConkey's classification and differential tests were meanwhile awakening much interest and discussion. Jackson (1911), in America, while believing that the presence of any types of coliform bacteria in water or milk indicated faecal contamination, proposed adding raffinose and mannite to the fermentation tests recommended by MacConkey. Savage (1912), in England, stressed the importance of correlating the various distinctive properties with environment. "Unless," he said, "the use of elaborate classifications of B. coli shows a definite distribution in Nature and a different significance, it cannot be said that these tests have any special value from the sanitary point of view." Howe (1912) demonstrated the absence of correlation between certain features, e.g., motility, fermentation of dulcite and mannite, indole production and nitrate reduction. He therefore distinguished only 2 types of coli: B. communis (sucrose-negative) and B. communior (sucrose-positive). Mackie (1913) taking a broad view of the coliform group considered that lactose fermentation was no more important for differentiation than any other sugar reaction, and suggested including the paracolon bacteria as an integral part of the coliform group. Systematically there is much to be said in favour of this view, but it would introduce a number of complications in the sanitary assessment of waters, foods, etc., if the coliform group were thus expanded.

Browne (1914), investigating the production of acid by the B. coli group, showed that in fermentation reactions both the configuration and complexity of the molecule were important. He thus explained the correlation which had been found in the fermentation of sucrose and raffinose (Winslow & Walker, 1907). He also observed that the organisms of the B. coli group could produce more acid from simple than from complex carbohydrates.

Houston (1913) still clung to his "FL-AG-IN-AC" definition of B. coli, which was virtually the same as that used by him in 1901. The true B. coli, he contended, should develop fluorescence (FL) in neutral-red broth, give acid and gas (AG) in glucose and lactose, form indole (IN), and produce acid and clot (AC) in milk. Attention has already drawn to his sound dismissal of the sucrose and dulcite results as immaterial.

Kligler (1914) proposed revising MacConkey's

classification by substituting salicin for dulcite, as he had found that the sucrose-salicin results, as compared with the sucrose-dulcite results, correlated more highly with the indole, Voges-Proskauer and gelatin reactions. His groups are aligned with those of MacConkey in Table 18.

TABLE 18.

Suggested Fermentative Classifications for the Coliform Bacteria.

Group	MacConkey		Kligler			Representative Organism
	Sucrose	Dulcite	Sucrose	Salicin	(Dulcite)	
1	-	-	-	-	usually -	B. acidi lactici
2	-	+	-	+	" +	B. coli communis
3	+	+	+	-	" +	B. coli communior
4	+	-	+	+	" -	B. lactis aerogenes

The disadvantages of accumulating fermentation reactions and of making fine distinctions which resulted in a multitude of strains were better appreciated by Prescott and Winslow (1915) who decided that the correlation of characters should be taken into consideration and that the coliform organisms should be classified on a statistical basis.

The most important work of this period was, indeed, done in America where the value of the gas ratio had all along been more fully recognized. It will be remembered that (1) Theobald Smith had associated a large gas volume and a gas ratio (CO_2/H_2) greater than unity with B. aerogenes and B. cloacae, as against a smaller volume and a lower ratio in the case of B. coli; (2) Russell and Bassett (1899) had indicated a high gas ratio to be a feature of the soil coliforms; (3) Durham had shown aerogenes and similar polysaccharide fractors to be Voges-Proskauer-positive; and (4) Howe (1904) had insisted on the value of the Voges-Proskauer test, correlating a positive reaction with the production of a large amount of gas. We have also seen how MacConkey (1905) unwittingly obtained a perfect correlation between the Voges-Proskauer reaction and the gas ratio in his classical strains, but tended to stultify his experimental results (1905-1909) by omitting or disregarding the Voges-Proskauer reactions and the gas ratios.

The gas ratio became the subject of close study by Rogers, Clark and Davies (1914) who, while allowing that the fermentation of a sugar was a reliable criterion, considered that by reason of secondary alkaline reactions the production of acid could not be accurately measured. They found, on the other hand, that no such disadvantage was attached to the determination of the gas ratio, as this remained constant under uniform conditions. Working with 124 coliform bacteria which they had recovered

from milk, and using a modification of the improved method of Keyes (1909), they estimated the gases produced by the organisms after growing in 1% glucose broth at 30°C. They found that though the total volume of gas varied considerably it was always composed of a mixture of hydrogen and carbon dioxide, and that the amount of hydrogen remained practically constant, the differences in the total volume being due to an increase in the amount of carbon dioxide. They confirmed Smith's (1895) conclusion that the gas ratio afforded a basis for classifying the coliform bacteria, and recognized three more or less well defined groups:

(1) 65 cultures gave a ratio of approximately 1,
(2) 24 " " " " " between 1·5 and 2,
(3) 35 " " " " " more than 2.

They also classified the organisms on MacConkey's criteria and showed that the low ratio types were to be found in each of MacConkey's "Groups", while the high ratio (over 1·5) types belonged mainly to MacConkey's "Groups" 3 and 4. They noticed that all the low ratio organisms failed to ferment starch, inulin and adonite, and were characterised by a large proportion which could form indole. They suggested that the name "B. coli" be applied to this class, reserving the name "B. coli communior" to those of the class which fermented sucrose and raffinose, and "B. coli communis" to those which did not. The organisms of the higher ratio class (CO_2/H_2 greater than 1·5), they noted, occupied the major part of MacConkey's "Group 4" but consisted also of 50% of the members of MacConkey's "Group 3". This class showed a much lower proportion of indole-forming organisms.

Rogers, Clark and Evans (1914) also studied 150 cultures obtained from bovine faeces. These were all non-liquefiers, and all but one formed indole and gave a low gas ratio (i.e., about 1). The one high ratio organism failed to produce indole. They compared these results with those obtained with milk, showing that in bovine faeces practically all the coliforms were of low ratio type, whereas of the coliforms found in milk some 50% belonged to the high ratio class. They inferred that the source of these latter must be sought elsewhere than in bovine faeces. To prove their contention, the same workers (1915) investigated 166 glucose-fermenters derived from dried grass and oats. Of these, 160 were coliform lactose-fermenters, of which only 8 belonged to the low ratio class, while 151 gave a ratio of 2 to 3 and 1 produced carbon dioxide alone. They therefore deduced that the high ratio type in milk might be derived from grasses and grains. They had thus gone some considerable way to confirm the observation of Russell and Bassett (1899) that the high ratio types belonged to the soil.

The estimation of the gas ratio was, however, still

a cumbersome procedure, and Clark and Lubs (1915), seeking a more convenient manner of distinguishing between the low and high ratio types, discovered a definite correlation between the gas ratio and the pH attained in a buffered solution of glucose. They used as their standard medium a solution containing 0·5% Witte's peptone, 0·5% glucose, and 0·5% dipotassium phosphate (K_2HPO_4), incubated the inoculated tubes for 3 to 5 days at 30°C., and then added methyl red as an indicator for demonstrating the pH reached. The low ratio coliforms, they showed, produced acid so actively as to stop their growth while much of the sugar was still unfermented, and the resultant solution remained acid to methyl red (pH of 4·5 or less) causing the indicator to become red. The high ratio organisms, on the other hand, did not produce acid so vigorously and were able to continue the glucose fermentation until all the sugar was exhausted; then by the formation of carbonates the acid became neutralised and a reversion to alkalinity occurred, the final pH being greater than 6 at which point methyl red turns yellow. By general consent the production in the glucose-phosphate medium of an acid or alkaline reaction to methyl red became known as methyl-red-positive and methyl-red-negative results respectively. Clark and Lubs had, therefore, established the important correlation between a low gas ratio and a methyl-red-positive reaction. At the same time they did acknowledge that there were occasions when doubtful tints were obtained.

Rogers (1916) joined these workers in attempts to correlate this reaction with the source of the organism. Of 113 coliform cultures obtained from human faeces, only 6 gave a negative methyl-red reaction, whereas of 137 cultures obtained from surface water 90 were methyl-red-negative. The conclusion reached was that the methyl-red-pos. types were occasionally found in springs which presented no evident source of contamination, but were especially abundant in polluted waters.

Levine (1916, a, b, c) investigated both the methyl-red (hereinafter MR) and Voges-Proskauer (VP) tests in a number of coliform bacteria which he obtained from various sources, and found an almost perfect negative correlation between the two reactions, i.e., practically all organisms which gave a positive MR result gave a negative VP reaction, and vice versa. He also noted that there was a relationship between these reactions and the source of the organisms. Thus, 117 strains obtained from human and animal faeces were all MR+VP-, whereas of 39 cultures from sewage 30 were MR+VP- and 9 were MR-VP+. In addition to these 156 organisms, he investigated (1916a) 31 strains obtained from other workers and found 12 MR+VP- and 19 MR-VP+, thus continuing to prove the negative correlation. He addmitted, however, that there were cases where it was difficult to decide whether the VP reaction was negative or positive. In studying this test (1916b) he showed

that organisms which gave a positive reaction in glucose did likewise in all other sugars which they fermented, and that those organisms which gave a negative result in glucose behaved similarly in all other sugars, except for maltose, from which most coliform bacteria can liberate a trace of acetyl methyl carbinol.

Hulton (1916) also found the same relationship between the MR and VP tests in the case of 45 coliform strains from various sources. Thus, 12 cultures from human and rabbit faeces were all MR+VP-, whereas of 33 strains from milk, water, sewage, urine and egg powder only 11 were MR+VP- while 22 were MR-VP+.

Greenfield (1916) obtained similar results with 432 coliform organisms (recovered from surface and ground waters, and natural and artificial ice) of which 138 were MR-. All these gave a positive VP reaction, while those of the MR+ cultures all gave a negative VP result.

The first evidence that the correlation between the MR and VP tests was not quite perfect was produced by Johnson (1916) who examined 363 coliform organisms from soil and found 42 of these to give MR-VP- reactions. Of the remaining 321 strains, 102 were MR+VP- while 219 were MR-VP+. She inferred from the high proportion (72%) which were MR-ve that the prevailing soil coliform was of the B. lactis aerogenes type. Burton and Rettger (1917) also found that the predominant coliform in soil was of the VP+ve type, but that many were of the liquefying (B. cloacae) variety. Of 193 non-sporing lactose-fermenters obtained from soil only 19% were VP-ve while 76% were VP+gel+ and 5% VP+gel-. They investigated the MR.VP relationship and concluded that the low ratio organisms remained consistently MR+VP-, but that the high ratio types seemed to give an inconstant pH at any given time and so became either VP+MR- or VP+MR+.

This question was further pursued by Johnson and Levine (1917), Levine, Weldin and Johnson (1917), Levine (1918), and Rogers, Clark and Lubs (1918). In all cases an almost perfect inverse correlation was found. The delay experienced in awaiting the VP result stimulated Levine, Weldin and Johnson (1917) to devise an accelerating process by the use of hydrogen peroxide, but this did not appear to receive general approval. Levine (1918) concluded that the coliform organisms should be subdivided on correlated characters. He elaborated a scheme of classification whereby the lactose-fermenters were subdivided on the MR,VP tests and fermentation of starch into (1) an aerogenes-cloacae group - giving a MR-VP+ result or fermenting starch, and (2) a coli group - giving a MR+VP- result and failing to ferment starch. Each group was then subdivided on biochemical features and motility, the correlation of characters being taken into considera-

tion. He quoted the relative proportions of coliform strains in soil as:

cloacae	49·7%,
aerogenes	30·5%,
MR+ types	18·7%,

i.e., somewhat in line with the results of Burton and Rettger (1917). He noted that as many as 25% of the MR- organisms were indole-positive.

Rogers, Clark and Lubs (1918) applied the 3 tests, gas ratio, MR and VP, to 177 coliform organisms which they had obtained from human faeces. All were non-liquefiers, and 131 gave a gas ratio of 1·06, a positive MR and a negative VP reaction, 127 being indole-positive and 4 indole-negative. The other 46 strains gave a gas ratio of between 1·5 and 2·7, a negative MR and a positive VP reaction. Only 10 were indole-positive. Thus, a perfect correlation between all three tests was obtained. The rather large proportion of high ratio types is partially explained by the facts that special methods of isolation were used and that 31 of the VP+ organisms were recovered from a single specimen of faeces. In this paper they also observed that the majority of MR- strains derived from grains did not ferment adonitol and that the MR- types tended to outgrow the MR+ types in milk which was allowed to curdle at 20°C.

Bronfenbrenner and Davis (1918), having isolated a number of late-lactose-fermenters from various foods, found that they could induce the production of gas within 24 hours by continual transference of the culture in lactose-peptone-water or by increasing the concentration of lactose to more than 1%. This observation emphasises the vagueness of the outer limits of the coliform group and adds weight to Mackie's (1913) almost justifiable proposal to include the paracolons.

The clear demarcation of the coliform bacteria on the MR and VP tests having been recognized, the relative viability of the two types became the next subject of investigation. Cohen (1918) proved the greater viability of B. aerogenes in stored water; Rogers (1918) also showed that B. aerogenes types could survive longer than B. coli in (1) water held in bottles, (2) running water, and (3) polluted streams. Savage (1918) by experimental methods showed that VP+ organisms could live for over 3 years in hard water which had previously been heated. Winslow and Cohen (1918) also noted the greater viability of aerogenes types in water. They showed that MR- types increased after 9 weeks' storage from 46% to 71%, but did not find that the proportion of such types was greater in naturally unpolluted or stored waters than in polluted or unstored waters. They admitted the occasional occurrence of doubtful MR tints.

Wood (1918) also investigated the viability of the VP+ coliforms, and (1919) recorded the natural persistence of aerogenes types in deep well waters obtained from the Limestone. He was the first British worker to make use of the MR test and applied it and the VP test to 471 coliform organisms isolated from human and animal faeces, cereals, grains, milk, and water. He also found the almost perfect inverse correlation described by the Americans, and obtained the results shown in Table 19.

TABLE 19.

The relative frequency of coliform bacteria according to the methyl-red reaction (Wood, 1919).

Source	Total No.	MR+		MR-			
				Indole-		Indole+	
		No.	%	No.	%	No.	%
Human faeces	33	33	100	0	0	0	0
Animal faeces	99	91	92	4	4	4	4
Milk	93	77	83	14	15	2	2
Water	231	154	67	65	28	12	5
Cereals & grain	15	4	26	10	67	1	7

He concluded that the MR-VP+ organisms were rare in faeces, commoner in sewage and surface waters, and the predominant type in grain and soil. He, therefore, assumed that they were the natural survivors of the faecal lactose-fermenters or were derived from soil or possibly grain.

Koser (1918) showed that B. lactis aerogenes could utilise uric acid (and other organic substances containing combined nitrogen) as the sole source of nitrogen, and could thus grow and multiply in a peptone-free medium so long as uric acid was present. B. coli and allied forms were unable to develop in such a medium. He investigated altogether 50 strains of B. lactis aerogenes and 74 coli strains, and noted this distinction in all cases. He also recorded that 2 of his "coli" strains gave MR-VP- reactions.

Chen and Rettger (1920) added this test to those already in use in a correlation study of 467 coliform bacteria which they isolated from soil and compared with 173 cultures obtained from human and animal faeces. The faecal strains were all gelatin-negative, MR+VP-, indole+, and uric-acid-negative. Of the soil coliforms, 20 were gelatin-negative, MR+VP-, 15 being indole+ and 5 indole-, and 10 being utilisers of uric acid and 10 non-utilisers. In other words, 193 B. coli types had been obtained, of which 10 utilised uric acid and 5 failed to form indole. The remaining 447 soil strains were all MR-VP+, and uric-acid-positive. They all failed to liquefy gelatin at

20°C. (though 17 achieved liquefaction at 37°C.) and were (roughly speaking) aerogenes types. Of these, 141 formed indole and 306 did not. In the course of the investigations 18 MR+VP+ strains were obtained, some of which resumed normal reactions on replating. If we allow "UA" to stand for uric acid, these results may be summarised as follows:-

TABLE 20.

Characters of the coliform bacteria isolated by Chen and Rettger (1920)

Source	Total	Number showing these reactions	Type
Faeces	173	173 Gel-MR+VP-UA-indole+	B. coli
Soil	447	10 Gel-MR+VP-UA- 10 Gel-MR+VP-UA+ 427 Gel-MR-VP+UA+	B. coli ? B. aerog.

These workers noted that they were unable to establish any correlation between indole-formation and the other reactions.

Despite the emphasis laid by most bacteriologists at this stage upon these new tests, some of the American workers were still concentrating on fermentation reactions. Thus, Winslow, Kligler and Rothberg (1919) still persisted in Kligler's (1914) salicin modification of MacConkey's classification. They stressed the importance of salicin even to the extent of presuming to alter the dulcite and adonite reactions of such classical strains as B. grünthal, B. neapolitanus and B. coscoroba (vide Suckling, 1943, p. 457).

Summary and Discussion
(1910-1920)

In the period 1885-1905 it had been realised that the prevalent coliform organisms in faeces were of the B. coli type, but it had also been noticed that similar organisms were to be found in sites, such as soils and waters, whose liability to faecal contamination was extremely improbable. That the principal soil coliforms differed from the faecal B. coli was observed as early as 1899 by Russell and Bassett who used the gas ratio to make the distinction. Neither this nor the Voges-Proskauer test received, however, adequate attention by subsequent workers who, if they used these tests at all, regarded them as of no greater importance than any one of the numerous fermentation reactions. It has been shown that MacConkey barely employed the VP test in his early (1905) attempts at classification and that in his latest (1909) work it still received insufficient emphasis, while the gas ratios were omitted altogether owing

to the mistaken belief that they had been proved unreliable (Longley and Baton, 1907). Some attempt has been made to show what very effective conclusions MacConkey could have drawn had this point been properly appreciated. In his latest article (1909) MacConkey, when considering the relative frequency in different sites, placed practically no stress on his 4 groups, but tended rather to regard each strain separately. It might almost be hazarded that MacConkey had himself realised that his method of classifying by multiple fermentation tests led not to a subdivision into groups which could be correlated with source (which was what he had hoped to achieve) but to a multiplicity of individual strains.

It is certain, however, that Rogers and his co-workers had this in mind when they commenced their investigations into the gas ratio; that they shared with Savage (1912) the view that until results could be correlated with habitat the sanitary value of ever-increasing differentiation was negligible. Success crowned their efforts for they not only found the gas ratio to be a reliable criterion but proved its correlation with source, thus confirming the work of the very early investigators. The low-ratio coliforms were shown to be particularly common in faeces, whereas the high-ratio types were prevalent in dried grass and grains.

But for the Great War, the next step would, no doubt, have been the correlation of the high gas ratio with the positive Voges-Proskauer reaction (as had been inadvertantly demonstrated by MacConkey, 1905). The English workers were, not surprisingly however, otherwise engaged during these years, and the Voges-Proskauer test had not been popularly accepted in America. Instead, the next step was the elaboration of the methyl-red test (Clark and Lubs, 1915) and the correlation of a positive result with a low gas ratio. Finally, in 1916 (Levine) it was shown that all three tests worked in parallel and that they all showed the same correlation with source which Rogers et al (1915) had propounded. These facts were abundantly confirmed in the subsequent four years.

The individual findings are chronologically tabulated (after the style of Bardsley, 1926) in Table 21, while in Table 22 the sources are grouped and percentage figures worked out on the combined results. The preponderance of the low-ratio coliforms in faeces and of the high-ratio types in soil, grass, etc., is obvious.

By 1920, therefore, the fundamental importance of these tests was generally appreciated both in England and America as affording the first reliable distinction between "faecal" and "soil" coliforms, and on this basis the relative viability of the two groups was investigated. All the evidence went to prove that the high-ratio types

TABLE 21.

Characters of coliform bacteria in various sites (1908-20)

Reference	Source of strains	No. of strains	CO_2/H_2		MR		VP		Indole		Gelatin	
			2	1	+	-	+	-	+	-	+	-
Ferreira et al, 1908	Human faeces	117	-	-	-	-	8	109	116	1	0	117
MacConkey, 1909	Human faeces	178	-	-	-	-	11	167	163	15	3	175
	Animal faeces	154	-	-	-	-	8	146	145	9	7	147
	Cereals & foods	71	-	-	-	-	32	39	27	44	35	36
Clemesha 1912	Human faeces	1207	-	-	-	-	64	1143	-	-	12	1195
	Cow faeces	1029	-	-	-	-	139	890	-	-	104	925
Rogers et al., 1914	Milk	124	59	65	-	-	-	-	-	-	-	-
1914	Bovine faeces	150	1	149	-	-	-	-	149	1	0	150
	Grass & oats	160	152	8	-	-	-	-	-	-	-	-
1916	Human faeces	113	-	-	107	6	-	-	-	-	-	-
	Surface water	137	-	-	47	90	-	-	-	-	-	-
Levine, 1916	Faeces	117	-	-	117	0	0	117	-	-	-	-
	Sewage	39	-	-	30	9	9	30	-	-	-	-
	Other workers	31	-	-	12	19	19	12	-	-	-	-
Hulton, 1916	Faeces	12	-	-	12	0	0	12	11	1	2	10
	Milk, etc.	33	-	-	11	22	22	11	16	17	15	18
Greenfield, 1916	Water	432	-	-	294	138	138	294	-	-	-	-
Johnson, 1916	Soil	363	-	-	102	261	219	144	-	-	-	-
Burton & Rettger, 1917	Soil	193	-	-	-	-	156	37	-	-	147	46
Rogers, et al., 1918	Human faeces	177	46	131	131	46	46	131	137	40	0	177
Wood, 1919	Human faeces	33	-	-	33	0	0	33	-	-	-	-
	Animal faeces	99	-	-	91	8	8	91	-	-	-	-
	Milk	93	-	-	77	16	16	77	-	-	-	-
	Water	231	-	-	154	77	77	154	-	-	-	-
	Cereals & grain	15	-	-	4	11	11	4	-	-	-	-
Chen & Rettger, 1920	Soil	467	-	-	20	447	447	20	156	311	17	450
	Faeces	173	-	-	173	0	0	173	173	0	0	173

TABLE 22.

Distribution of coliform organisms in various sites.
(Results shown in Table 21 grouped as to source)

Source	Total No. of strains	Low ratio, MR+ or VP-		High ratio, MR- or VP+	
		No.	%	No.	%
Faeces	3559	3268	91·8	291	8·2
Human faeces	1825	1690	92·6	135	7·4
Animal faeces	1438	1276	89·1	156	10·9
Water	872	536	61·5	336	38·5
Milk	250	92	36·8	158	63·2
Soil & grains	1269	210	16·5	1059	83·5

were more resistant than the typically faecal organisms. The relative unimportance of the differential fermentation reactions was recognized by all but a few workers (notably Kligler) and the general view that characters must in future be correlated and classifications made on a statistical basis was, perhaps, best expressed by Prescott and Winslow (1915).

During the same period doubts were voiced in some quarters as to whether the fermentation of lactose was a fundamentally necessary attribute of the coliform bacteria. Mackie (1913) was strongest in this view, and Bronfenbrenner and Davis (1918) had demonstrated that the ability to ferment lactose rapidly could be developed in late-lactose-fermenters. Not only, therefore, was internal classification being rearranged but the outer limit was also subject to discussion.

Finally, in 1918 a further characteristic of the high-ratio types was discovered by Koser (1918) - the ability to utilise uric acid as the sole source of nitrogen; while in 1920 (Chen and Rettger) this power was shown to be also possessed by a small number of the low-ratio types (vide Table 20).

As compared with the period immediately preceding, there had, therefore, been a complete swing of opinion away from fermentation reactions and towards a series of tests which correlated one with another and with the expected degree of faecal contamination. In fact, correlation may be said to have been the motto of this decade, and a great many observations combined to prove the existence of two groups of coliform bacteria, one predominant in faeces (gas ratio of about 1, MR+, VP-), and the other (gas ratio of about 2, MR-, VP+) prevalent in the soil. The distinction was not altogether clear-cut, however, for small numbers of the second group were always present in faeces, and the faecal types could also be found in the soil. Moreover, the tests themselves did not always agree absolutely; some dubious MR tints, some doubtful VP reactions occurred and there were occasions when a double negative or a double positive reaction was obtained. The work of Koser (1918) and Chen and Rettger (1920) further indicated that the coliform group did not altogether clearly divide on the MR and VP tests but that there were still some complexities to be explained.

The Characters and Distribution of the Coliform Bacteria.

HISTORY AND LITERATURE

Period 1920 - 1934

Considering the part played by Levine (1916,1918) in proving the correlation between the gas ratio, the methyl-red test and the Voges-Proskauer reaction, it is not surprising that he became a whole-hearted supporter of the new method of subdividing the coliform group. He collected together the results of all the other workers who had made investigations similar to his own, and allocated to the B. coli class all organisms which were found to give a low gas ratio, a positive MR test, or a negative VP reaction, and to the B. aerogenes class all organisms giving the opposite results. In 1921 he published the results shown in Table 23. On comparing this with Table 22, which was compiled in the same way, it is to be noted that the figures are substantially the same though those of Levine approach a little farther towards 100%.

TABLE 23.

The correlation of coliform types with habitat
(Levine, 1921)

Source of strains	No. of strains examined	B. coli types %	B. aerog. types %
Human faeces	2,534	94·1	5·9
Animal faeces	1,832	92·6	7·4
Faeces	4,366	93·5	6·5
Soil, grains, etc.	1,141	13·5	86·5

Levine was also fully aware of the disadvantages of the fermentative method of differentiation. By using 10 uncorrelated tests, he pointed out, one could prepare to meet over 1,000 different combinations. He also (1921a) reiterated his belief in the importance of the Voges-Proskauer test.

Mackie (1921), approaching the problem from a totally different angle, placed emphasis on the indole test, and the fermentation of inositol, as criteria for the classification of coliform bacteria. He still maintained his view (1913) that the fermentation of lactose was relatively unimportant, and considered that the indole and inositol reactions were of more consequence than the lactose or any other "sugar" reaction for differential purposes. He proposed that all the saprophytic glucose-fermenting organisms of the coli-typhoid group should be

regarded as coliform bacteria, irrespective of their other fermentation reactions, and classified into sub-groups on the following basis:

TABLE 24.

Classification of the coliform group.

(Mackie, 1921)

Sub-group	Prod^{n} of gas	Form^{n} of indole	Ferment^{n} of inositol	Liquef^{n} of gelatin	e.g.
A	+	+	-	-	"typical" B. coli
B	+	-	-	-	(Bact. coli, type II)
C	+	±	+	-	B. lactis aerogenes
D	-			-	B. coli anaerogenes*

*a curious type which produces acid without gas in the sugars usually fermented.

Mackie also included in the coliform group certain non-proteolytic glucose-fermenting saprophytes which resembled B. coli in their general characters, but which did not ferment lactose (unless after mutation), i.e. the 'paracolon' bacteria. He found that strains having the characters common to the coliform group (as defined by him) did not liquefy gelatin (in 2 weeks at 22°C.) apart from some strains which could be classed as B. proteus. His views in this respect are reminiscent of the observations of Jordan (1903). He confirmed the findings of MacConkey (1905) in regard to the wide fermentative powers of the lactose-fermenters which, he showed, fermented also, as a general rule, fructose, galactose, maltose, dextrin and glycerol.

Mackie stressed the importance, however, of inositol and pointed out that fermentation of this sugar was correlated with other characters, e.g., non-motility, encapsulation, production of large, thick, opaque, slimy colonies, and fermentation of lactose, adonitol, sucrose, raffinose and salicin. In this article, also, he noted the occurrence of strains of B. friedlanderi in faeces.

An important finding, in view of Mackie's opinion regarding lactose-fermentation, was that of Dudgeon (1924) who observed that, in 49 cases of unusually severe B. coli infections of the urinary tract, all the strains isolated were of a slow lactose-fermenting type.

The survival of B. coli in water was studied by Winslow and Falk (1923) who showed that pH was an important factor. They added known numbers of B. coli to samples of sterile, distilled water (of various pH) which were then incubated at 37°C., and investigated the per-

centage of organisms surviving after 9 hours. Their results are shown in Table 25.

TABLE 25.

The effect of pH on the survival of B. coli in distilled water.

(Winslow & Falk, 1923)

pH	4	5	6	7	7·5	8
% surviving after 9 hours at 37°C.	1	82	106	54	35	12

A further method of testing for indole was introduced by Holman and Gonzales (1923) who credited it with greater delicacy. The method took advantage of the volatility of indole at 37°C. and consisted in placing a strip of filter paper, impregnated with oxalic acid, in the upper part of the tube before incubation. On the production of indole the paper turned pink. Subsequent workers (notably Bardsley, 1926) proved this method to be a little more sensitive than the Böhme (or Erlich) test, and it is one of the standard methods in use at the present day.

The greatest contribution of this period was without doubt that of Brown (1921) who made the suggestion that a synthetic citrate medium might be of greater use than the uric acid medium for distinguishing soil coliforms from faecal coli. This proposal was put into effect by Koser (1923) who devised a medium which contained:

sodium citrate	2	gm.,
sodium ammonium hydrogen phosphate	1·5	gm.,
potassium dihydrogen phosphate	1	gm.,
magnesium sulphate	0·2	gm.,
distilled water ad	1000	c.c.,

and nothing else. The citrate was thus the sole source of carbon, just as the microcosmic salt provided the only nitrogen. Koser found that the aerogenes-cloacae types (MR-VP+) could grow in this medium and produce a visible turbidity, whereas the B. coli types of faecal origin were unable to do so, the tube remaining clear. A further distinction had thus been introduced to confirm the MR and VP differentiation.

Koser then (1924, 1924a) applied this as an additional test to a number of coliform organisms isolated from faeces, soil and water, and made a further discovery: most of the MR+ strains from soil (see Table 26) were also able to grow in the citrate medium. A means had, therefore, become available for distinguishing the soil "B. coli types" from the truly faecal B. coli. The former (MR+VP-citrate+) became known as "Intermediate" coliform bacteria, i.e., resembling true B. coli in some re-

spects (MR+VP-) and the aerogenes-cloacae types in others (e.g., citrate+).

TABLE 26.

Characters of coliform bacteria found in soil and faeces (Koser, 1924)

Source	No. of strains	B. coli MR+VP-cit-		Intermediate MR+VP-cit+		B. aerog. MR-VP+cit+		Irregular cit+ strains	
		No.	%	No.	%	No.	%	No.	%
Faeces	118	107	90·7	1	0·8	9	7·6	1	0·8
Soil	72	2	2·8	16	22·2	36	50·0	18	25·0

Applying this test to a series of known polluted and unpolluted waters, in which the percentages of MR+ organisms were approximately the same, Koser (1924a) obtained the results shown in Table 27.

TABLE 27.

Proportion of citrate-positive organisms in various waters (Koser, 1924a)

Source: Water	No. of strains	Citrate- %	Citrate+ %
Polluted	107	64·5	35·5
Unpolluted	90	16·7	83·3

The citrate test had, therefore, very considerably reduced the discrepancy between the bacteriological results and the indication of the sanitary surveys.

Alive to the possibility that the power to utilise citrate might be a character acquired by faecal coli as the result of unfavorable environment, Koser experimented with strains of B. coli and B. aerogenes. He inoculated both into sterile water, B. coli into sterile soil, and B. aerogenes into sterile faecal suspensions, and examined them after several months. He also examined strains which had been subcultured in artificial media. His conclusion was that "the ability to utilise citrate is apparently a fairly stable character and evidently is not readily acquired or lost."

Another important observation made by Koser (1924a) was that the majority of the citrate-positive coliforms (in soil, at least) were indole-negative. Pawan (1926) also discovered a close agreement between growth in citrate and failure to produce indole in coliform organisms recovered from river water, and (1925, 1926) confirmed Koser's findings in all other respects. He proved the comparative absence of citrate-utilising coliforms from faeces (e.g. 3·7%), the small proportion (9·1%) in polluted water, and the high proportion (81 to 90%) in unpolluted sites (water and soil). His findings are tab-

ulated in Table 28.

TABLE 28.

Proportion of citrate-positive coliforms in various sites.
(Pawan, 1925 and 1926)

Source	No. of strains tested	Citrate-reaction	
		Negative %	Positive %
Faeces	432	96·3	3·7
Polluted water	210	90·9	9·1
Unpolluted water	240	18·7	81·3
Unpolluted soil	214	10	90

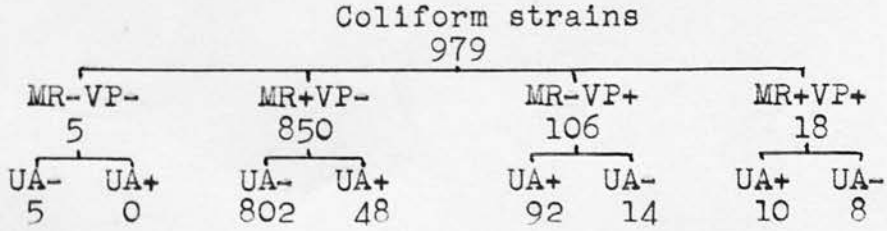
As an example of the closer correlation which the citrate test gave with source, it is noteworthy that, while 42·5% of the 240 strains isolated from unpolluted water were MR+, only 18·7% were citrate-negative.

Brown (et al., 1924) was also responsible for suggesting a further selective medium - tartrate peptone water - to reinforce the citrate distinction, but this did not gain popular acceptance. Another test which failed to obtain permanent general approval was Koser's uric-acid-utilisation. This was no doubt due to its being so quickly followed by the citrate test which gave a better correlation with source and to which attention and energy were almost at once deflected. The uric acid medium did, however, receive a fair trial in the work of Bardsley (1926) who applied it together with the MR and VP tests in an investigation of coliform organisms recovered from water. She found it to give only an imperfect correlation with the other reactions.

Bardsley's investigation comprised the examination of 525 potable waters and extended over a period of 18 months. Altogether 1441 coliform strains were isolated and proved to be Gram-negative, non-sporing bacilli which gave acid and gas in lactose and acid and clot in milk. All were submitted to the indole, MR, VP and gelatin (5 days) tests. The uric acid test was applied to the first 979 strains, but was thereafter discontinued for the reason mentioned above. The results obtained with these 979 strains may be of interest and are graphically represented in Table 29.

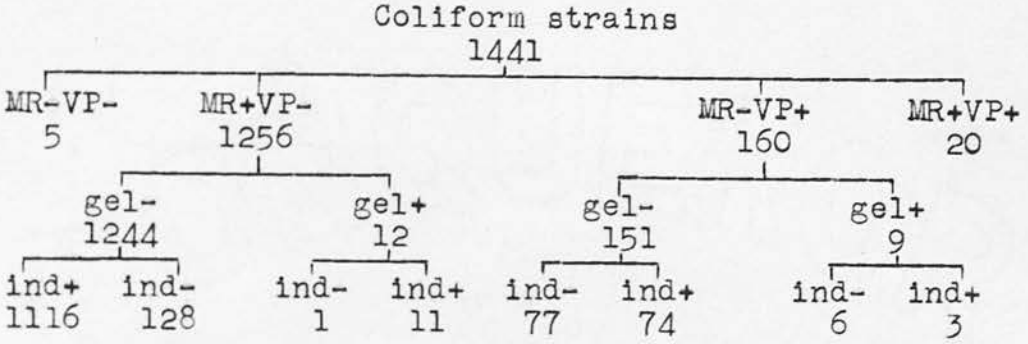
TABLE 29.

Characters of coliform strains from water
(Bardsley, 1926)



Bardsley's final results are similarly represented in Table 30.

TABLE 30.
(Bardsley, 1926)
Characters of coliform strains from water.



Bardsley drew attention to the almost complete inverse correlation between the MR and VP results but noted that in a small percentage (1·7%) of cases a double positive or double negative reaction was obtained. She pointed out that similar anomalies had been met with by previous workers (Clark and Lubs, 1915; Johnson, 1916; Burton and Rettger, 1917; Koser, 1918; Rogers et al., 1918; Winslow and Cohen, 1918; Chen and Rettger, 1920) and was convinced that such abnormal types did, in fact, exist though in very small proportions.

Having summarised the literature to prove the greater faecal significance of the MR+VP- types, she showed that without the use of these tests 1395 (1244 + 151) of the total 1441 strains would be classified as B. coli on the lactose, milk and gelatin tests, whereas on consideration of the MR and VP reactions 151 (or 11%) of these proved to belong to the aerogenes type and were therefore "of no significance as indicators of faecal pollution." Hence, the application of the MR and VP tests made "a practical difference in the interpretation of results in the bacteriological examination of water." This argument was reinforced when the results were regarded from the view-point of the samples themselves. Thus, of the 525 waters examined, 262 were found to contain coliform organisms, but, after differentiation, 6% of these latter proved to contain only MR-VP+ types and were therefore "probably associated with pollution from soil washings rather than contamination from faecal sources."

Before leaving this article, the examination procedure is worthy of notice. The samples received comprised all the various types (raw, treated, etc.) usually submitted for routine bacteriological examination. In each case varying amounts of the sample (after shaking) were added to tubes of MacConkey bile-salt lactose peptone medium, and primary incubation carried out for

24-48 hours at 37°C. Of the tubes showing the production of acid and gas, that representing the smallest volume of the sample to give a positive reaction was subcultured to MacConkey agar, acid colonies therefrom being submitted to the obligatory and differential tests. There is no stated assurance, however, that, in the event of all the colonies proving to belong to the aerogenes type, the tubes representing the larger volumes were tested for the presence of coli strains before the sample as a whole was considered to contain only aerogenes. Unless this procedure was actually adopted, the conclusions reached are invalid; but it is more than likely that this precaution was taken, for in a later article (1934) Bardsley specifically mentions it as part of the routine. While any bacteriological examination of water - whether for routine or research purposes - must resolve itself into a compromise of some kind, there is in my opinion a considerable disadvantage (if only in loss of time) in concentrating too much upon the smallest volumes which show evidence of coliform bacteria. A representative selection of all the positive tubes gives a much better cross-sectional picture.

It is of material interest also that Bardsley classified 961 of her strains on the sucrose-salicin basis recommended by Kligler (1914 onwards) but found the results obtained to have little apparent significance. She concluded that the differential fermentation reactions, while useful for purposes of systematic classification, were of no value in assessing the probability of excretal pollution. Finally, she suggested that in the bacteriological examination of water, organisms should be regarded as significant which were of typical morphology and staining reaction, produced acid and gas in lactose, failed to liquefy gelatin, clotted milk and gave a positive MR and a negative VP reaction. While allowing that indole-production added weight to the above characters, she felt that the absence of this power did not provide a sufficient basis for the exclusion of an otherwise typical bacillus from the "B. coli group."

The one test (citrate-utilisation) which Bardsley had omitted in her (1926) investigation was being further studied by other workers in the same year. The fermentation of cellobiose, a β-glucoside, had been shown by Jones and Wise (1926) to correlate with the MR and VP reactions, and Koser (1926a & b) proved that this property ran closely in parallel with the ability to utilise citrate. Conducting further investigations into the occurrence of citrate-positive coliforms in various sites, Koser (1926 and 1926c) obtained the results shown in Table 31. It is noteworthy that all the citrate-negative organisms gave the typical reactions of B. coli. Under the title "Irregular" are grouped certain citrate-positive strains which gave abnormal MR and VP reactions. In the final column are collected all the citrate-positive types.

TABLE 31.

Proportion of citrate-utilising coliform types in faeces and various soils

(Koser, 1926 and 1926c)

Source	Total no. of strains	Cit-neg.	Citrate-positive			
		B. coli %	Intermediate %	B.l.aerog. %	Irregulars %	Total %
Faeces	79	96·2	0	3·8	0	3·8
Soil	162	11·1	17·9	64·2	6·8	88·9
Unpolluted soil	104	23·1	7·7	67·3	1·9	76·9
Polluted soil	33	63·6	3·1	33·3	0	36·4

Cunningham and Raghavachari (1924, 1926) studied in India the proportions of the MR+ and MR- coliform types occurring in various sources, and Raghavachari (1926) introduced the citrate test in a series of soil and water examinations. He found the MR- type to be predominant in soil (cf. Tables 21, 23, 26 and 31) and the MR+ type predominant in water. A number of the latter (MR+) type recovered from filtered water supplies were citrate-utilisers which failed to form indole. He finally reported a complete inverse correlation between MR+citrate+ and indole-ve reactions among the 2092 strains which he obtained from both water and soil; or, in other words, not one of these organisms produced the reactions: MR+citrate+indole+. Actually, 54 samples of soil yielded only 8, and 158 samples of water only 63, Intermediate coliforms. His results are set out in Table 32.

TABLE 32.

Proportion of coliform types in soil and water.

(Raghavachari, 1926)

Source	No. of samples	No. of strains tested	B. coli		Intermediate		B. lactis aerogenes	
			No.	%	No.	%	No.	%
Soil	54	578	26	5·0	8	1·5	484	93·5
Filtered water	50	500	253	50·6	55	11·0	184	38·4
Unfiltered water	108	1074	743	69·2	8	0·7	323	30·1

In his concluding remarks, Raghavachari mentions that he did not consider it advisable to disregard altogether the MR+citrate+ types in assessing the purity of water. In view of the findings of the other workers, it is noteworthy that Raghavachari obtained no Irregular types among 518 soil strains.

The value of the citrate test was investigated by Hicks (1927) in Shanghai, where the MR, VP and indole tests had largely failed to distinguish faecal from non-faecal contamination of water supplies. He confirmed the sparsity of citrate-positive coliforms in faeces as he obtained only 11 (7·3%) utilisers among 150 strains col-

lected from human and animal faeces, whereas 40 out of 50 soil strains were citrate-positive. The faecal strains yielded 89·4% with the reactions typical of true coli (indole+MR+VP-citrate-), whereas 68% of the soil strains were indole-negative. Hicks' results are tabulated in Table 33.

TABLE 33.

Examination of 200 organisms of the coli-aerogenes group. (Incubation 5 days at 37°C. Figures are percentages) (Hicks, 1927)

Source of strains	No. of strains	Citrate		Indole		MR+ VP-	MR- VP+	MR± VP-	MR- VP-	Cit+ Ind-
		+	-	+	-					
Human faeces	100	7	93	92	8	95	1	3	1	7
Animal faeces	50	8	92	90	10	96	0	0	4	6
Faeces	150	7·3	92·7	91·3	8·7	95·3	0·7	2	3·3	6·7
Soil	50	80	20	32	68	76	20	0	4	66

While the MR and VP tests showed the almost complete absence of the B. aerogenes types from faeces, they were not helpful in the case of the soil strains, for 76% of these were MR+VP-. Hicks decided that the indole and citrate tests were more valuable as criteria of the origin of coliform organisms, and suggested that "if only bacilli which are indole-negative and citrate-positive, in combination, are regarded as non-faecal, the error will be on the safe side."

(Mention must here be made that it had for long been realised that the coliform standards which were applied to British and American waters were too severe for waters in the warmer and Tropical countries, such as Africa, India, etc., where many waters with no apparent faecal association but with high coliform counts ("B. coli" by the old standards) were daily consumed without any ill-effect to health. In these countries the advent of the citrate test was particularly welcome as it demonstrated the majority of these "coli" organisms to be citrate-utilisers and therefore removed them from the category of immediate faecal indicators.)

Experimenting with the tests themselves, Hicks showed that 48 hours' incubation was sufficient for the establishment of definite MR and VP reactions. As a matter of interest he applied the citrate test to a number of non-lactose-fermenters. He obtained the following results:-

No growth	Growth in 2 days
B. typhosus	B. enteritidis (Gaertner)
B. paratyphosus A	B. paratyphosus B
B. dys. Flexner V	B. suipestifer
B. dys. Flexner Y	B. faecalis alcaligenes
V. cholerae	(B. paratyphosus C - 4 days)

W. James Wilson (1928) reviewed the advances recently made and the opinions put forward as regards the bacteriological examination of water. Referring to the quantitative nature of the coliform test, he noted that the standards were becoming more rigid, "now often requiring the absence of B. coli from 100 c.c. where some years ago its absence from 1 c.c. would have been considered adequate" (cf. Theobald Smith and Brown, 1893). For the presumptive test he preferred MacConkey's medium to the American brilliant green and other modifications. Having mentioned Houston's "Flaginac" standard for B. coli, Wilson considered the new differential (MR, VP and citrate) tests in the light of his own experience and a study of the literature. He found these helpful in deciding whether a coliform bacillus isolated from a water supply was, or was not, significant of excretal contamination. Thus:

(1) MR-VP+ strains are (he contended) certainly not of recent intestinal origin. Such strains invariably grow in Koser's citrate solution.

(2) MR+VP-citrate- strains are highly suspicious of contamination.

(3) MR+VP- strains "may or may not indicate contamination. Berry and Ey (1926) have found such strains frequently in wells which on a field survey appeared to be free from the possibility of pollution."

(4) MR+VP-citrate+ strains do not suggest recent contamination. They are usually indole-negative.

(5) "Slow lactose fermenters are of little sanitary significance. Berry (1926) found that such strains grew in Koser's citrate solution and that a sanitary survey of the wells from which they were derived precluded the likelihood of contamination."

Wilson also suggested that further assistance might be afforded by the use of cellobiose which "is fermented by B. lactis aerogenes and not by excretal B. coli." He argued that the fermentation of cellobiose by any strain which had given the reactions MR+VP-citrate- should exclude it from the category of "typical B. coli".

Wilson's conclusion is admirably sound: "On the whole ... the quantitative test for coliform organisms is the most important, but .. in certain circumstances a qualitative study of the organism assists in the interpretation of its significance."

So many workers were now studying the coliform group in its many aspects that only the principal observations can be indicated. Greer and his colleagues (1928) had investigated the presumptive coliform test in an effort to discover the causes of false presumptive reactions, i.e., the production of acid and gas in a primary tube from which no coliform bacteria could subsequently be isolated. They showed that the commonest

cause was the growth of anaerobes, particularly B. welchii. Thomson (1927) had suggested the addition of 0·2% of potassium hydrogen phosphate (K_2HPO_4) to MacConkey's medium as a means of reducing the number of false presumptive reactions, but subsequent workers (notably Bardsley, 1934) found this to be of no value, even up to 0·5% (at which strength it began to precipitate the peptone).

The occurrence of variable VP reactions had been investigated by Linton (1924), Georgia & Morales (1926), Paine (1927), Williams & Morrow (1928) and Ruchhoft, Kallas, Chinn & Coulter (1931) who showed that anomalous results could be produced by certain VP+ types which, in their fermentation of glucose, produced acetyl methyl carbinol as an intermediate product. A negative reaction in (say) 48 hours might, therefore, be followed in a further 24 hours by a positive, and finally a negative, result. The best procedure was, they stressed, to test subcultures of various ages.

A variation of Erlich's paradimethylamidobenzaldehyde reagent, in which amyl alcohol replaced the ethyl alcohol, was described by Kovacs (1928). That indole was extremely soluble in amyl alcohol had been noted by MacConkey (1909) but Kovacs' suggestion conferred a definitely increased sensitivity on Erlich's reagent.

The possible significance of the various coliform types was, perhaps, the most discussed problem. Ford (1927) stated, for instance, that B. lactis aerogenes occurred chiefly in bovine faeces. Cruickshank and Cruickshank (1931) considered the organisms of the B. lactis aerogenes, B. oxytocus and B. cloacae types all occurred in small numbers in most specimens of human faeces. Jordan (1928) and Ruchhoft et al. (1931) regarded B. oxytocus as merely an indole-positive variety of B. lactis aerogenes. Hill et al. (1929) isolated over 39% of aerogenes types (MR-VP+) among 200 Gram-negative bacilli from genito-urinary infections.

By a direct plating method, Tonney and Noble (1931) demonstrated the ratio of B. coli to B. aerogenes to be 100:1 in faeces, but 1:20 in soil and vegetation. They (1931a) found by experiment that under winter conditions B. coli and B. aerogenes, both from faecal material and from cultures, underwent rapid decline without showing any significant change in the relative numbers. These same workers (1932) proved by a sanitary survey of wells that the occurrence of aerogenes in the absence of coli is not necessarily associated with faecal pollution.

Organisms of the Intermediate type (VP-citrate+ indole-) were obtained from water by Lewis and Pittman (1928) and by Ruchhoft et al. (1931) who found they were extremely rare in human and animal faeces. Ruchhoft and his colleagues (1931) did occasionally find an indole-positive variety (VP-citrate+indole+) in surface water

and considered it was of soil rather than of faecal origin. They believed that many of the so-called Irregular strains were really mixed cultures (rather a poor tribute to the technique of the many other workers who had described the existence of such types), and drew attention to the fact that even the typical B. coli could grow slightly in the citrate medium.

Burke-Gaffney (1932) compared the results of some investigations he had made in Africa and Europe. He had studied the proportions of coliform bacteria present in faeces and soil in both continents, and decided that in Africa aerogenes was less common in faeces but more prevalent in the soil, whereas in Europe he found higher percentages of aerogenes in faeces and only small numbers in the soil, where the Intermediate types (VP-, citrate+indole-) formed a greater proportion of the total. In both countries the samples of soil were selected from sites subject only to remote pollution. He also made an extensive study of the waters both in Europe and the Tropics, grouping his sources under the terms "non-faecal", "partly faecal" and "wholly faecal" (see Table 34). As regards waters in Africa, he isolated MR+ coliforms from many waters of known purity but the majority of these were citrate-positive types. He believed that these Intermediates were typical of the soil and that their presence in water in the Tropics indicated, if anything, only remote pollution. In Europe, on the other hand, he never obtained coliform organisms from waters or soils which a sanitary survey had indicated to be free from pollution. The figures he obtained are shown in Table 34.

TABLE 34.

Relative proportions of coliform types in various sites.

(Burke-Gaffney, 1932)

Source	No. of strains tested	B. coli %	Intermediate %	B. lactis aerogenes %	Irregulars %
TROPICAL RESULTS					
Non-faecal (unpolluted water unpolluted soil)	653	8	12	76	4
Partly faecal (polluted water & soil cesspits, urine)	986	36	12	45	7
Wholly faecal	284	96	2	1	1
EUROPEAN RESULTS					
Non-faecal	-	-	-	-	-
Partly faecal	432	70	20	6	4
Wholly faecal	145	87	3	9	1

Gray (1932), echoing the views originally expressed by Kruse (1894), contended that coliform organisms were widely distributed and intermingled in nature and that aerogenes was as typical of the excretal flora as coli, although the latter type was present in greater numbers. He quoted some unpublished experiments of Cruickshank in which aerogenes had been recovered from 78 out of 135 samples of human faeces, and, by inoculating first into citrate, Gray himself obtained aerogenes from 37 out of 40 samples of faeces. Also, from 6 samples of soil - which were apparently unpolluted - Gray isolated aerogenes in every case, and coli from all but one. He studied the longevity of coli and aerogenes in water and confirmed the fact that the MR- types remained viable over a longer period than B. coli. He argued, therefore, that the presence of aerogenes in food or water meant nothing more than remote faecal pollution.

Kulp (1932) also experimentally studied the longevity of coli and aerogenes. He held 24 strains of the two organisms in soil culture for a considerable period. Six strains of coli and 2 of aerogenes survived for over $3\frac{1}{2}$ years and showed at the end of that time no evidence of change in the morphology, lactose-fermentation, MR or VP reactions, gelatin-liquefaction, or citrate and uric acid utilisation, but there was some variation in the indole reaction. Kulp concluded, therefore, that indole-production might be a variable characteristic (cf. Houston, 1901; Jordan, 1901; Horrocks, 1903; Savage, 1905, 1907; MacConkey, 1905).

Skinner and Brudnoy (1932) isolated 11% of citrate-positive coliforms among 585 strains from human faeces. They followed up the suggestion of Jones and Wise (1926) regarding the fermentation of cellobiose, but were unable to establish any correlation between this and the utilisation of citrate. They concluded that neither test was justified in water analysis. Koser and Saunders (1932) tested the growth of lactose-fermenters from soil and faeces in α-methyl-d-glucoside and decided that this medium also was of less value than citrate for distinguishing the Intermediate strains.

A further test - the production of trimethylene glycol from glycerol - was put forward by Werkman and Gillen (1932) for the differentiation of the Intermediate types (MR+VP-citrate+). They suggested that the coliform bacteria which possessed this property should be termed Citrobacter, and they recognised 7 distinct species. Levine et al. (1932) studied this test in an investigation of 401 coliform strains they had isolated from eggs. They classified these strains (11% of which were indole-negative Intermediates) on the basis of citrate-utilisation and the ability to produce trimethylene glycol. They further observed that the Intermediates obtained from eggs differed from other coliform types in producing H_2S.

Having isolated a number of slow lactose-fermenters from the faeces of cows suffering from infectious diarrhoea, Jones, Orcutt and Little (1932) tested 37 of these strains as regards sugar reactions, MR, VP, indole, H_2S and gelatin tests. All 37 strains gave the reactions typical of B. coli (MR+VP-indole+).

The importance of inositol-fermentation was stressed by Hay (1932) in a study of B. mucosus capsulatus (an inositol-fermenting coliform in all respects similar to B. lactis aerogenes - see Table 10, No. 103). He also noted the presence of small numbers of the friedlanderi and cloacae types in human faeces, and reported the occurrence of citrate-positive coli types (i.e. indole-positive Intermediates). In 1933 Malcolm embarked on an extensive investigation of the coliform flora of milk and bovine faeces, but this is more fully reported in later articles (1935, 1938). He also emphasised the importance of inositol for the purposes of classification.

Bamforth (1934) made an interesting enquiry into the coli-anaerogenes bacteria. Of the 5 groups which he recognized (Sonne, Dispar, alkalescens, etc.) only the B. coli anaerogenes itself need be described here. This he found to be biochemically distinct from the other groups and to produce acid without gas in 24 hours from lactose, glucose, maltose and mannite. Most strains formed indole.

The Committee on Bacteriological Technic (1934) added some light on the subject of gelatin-liquefaction. Misleading results, they stated, may be obtained on prolonged incubation owing to the action of endo-proteases liberated from dead bacterial cells.

Biggar (1934) and Bergey (1934) each had elaborated their own schemes for classifying the coliform group. Bergey divided the group into two genera on the basis of the VP reaction. The VP- genus he termed Escherichia (type species: E. coli ≡ typical B. coli) and subdivided this into 22 species, using motility, milk, nitrate-reduction, gelatin-liquefaction, and the fermentation of sucrose, salicin and dulcite. He gave the VP+ genus the name of Aerobacter and subdivided this into 7 species on motility, gelatin-liquefaction, gas-formation at 37°C., and the fermentation of sucrose and dulcite. This scheme, which is evidently an elaboration of that of Winslow, Kligler and Rothberg (1919), has not gained acceptance in this country, as altogether too much emphasis is laid upon the VP reaction.

Bardsley (1934) reported the results of a prolonged study of the coliform types she had isolated from water, soil, faeces and ice-cream. Having summarised the literature on the subject of the possible significance of the various coliform types she drew attention to the less rigid standards which were applied to waters in tropical

countries. There, where pollution was very heavy, the different types of coliforms were differentiated and only the true coli strains regarded as indicators of excretal contamination. By this means, the bacteriological analyses were brought into line with the findings from the sanitary surveys and with the known quality of the waters. "If more stringent standards were adopted," she stated, "many water supplies would be condemned which experience has shown to be safe for drinking."

The position in temperate climates, she felt, was not so well defined, and it had yet to be proved that classification on the basis of citrate metabolism correlated with "a different distribution in Nature and a different sanitary significance" (quoting Savage, 1912). She noted that some bacteriologists discounted the citrate utilisers in assessing the purity of water while others, having found these organisms in faeces and urine, insisted on their sanitary significance although, perhaps, their presence only indicated remote pollution.

She examined altogether 2144 samples of water by methods similar to those employed in 1926, except that the citrate test was now included. It is to be noted that only the tubes representing the smallest quantity of water to give a positive presumptive reaction were plated, and representative colonies thereafter selected and tested. If true B. coli (MR+VP-indole+UA-citrate-) were not isolated therefrom, the remaining positive primary tubes were plated out and further cultures tested. (N.B. If B. coli were obtained from the smallest volume, the flora of the larger volumes was evidently not studied. The emphasis was, as it should be, on B. coli, but this must be remembered when the proportions of the various types are expressed.) All organisms were tested for morphology, staining reaction, fermentation of lactose, clotting of litmus milk, and the following differential tests:

(1) production of indole (tested by the oxalic acid method),
(2) liquefaction of gelatin (glucose-gel. stab, 5 days, 20°C),
(3) MR and VP reactions (incubated 5 days at 30°C.),
(4) uric acid utilisation (Koser,1918; after 5 days at 30°C),
(5) citrate utilisation (Koser,1923; after 5 days at 30°C).

Of the 2144 samples of water examined 1102 gave a positive presumptive reaction and 4333 strains of coliform bacilli were isolated. These she classified as shown in Table 35. She noted that the correlation between the MR,VP and the 2 Koser tests was well defined, and also established a good correlation between the MR, Koser and indole tests. These are summarised in Tables 36 and 37. It is noteworthy that, despite her failure (1926) to obtain satisfactory correlations with the uric acid test, she nevertheless introduced it into the present study and was so well rewarded. It may be argued, however, that

TABLE 35.

Characters of coliform bacteria isolated from water.

(Bardsley, 1934)

Reactions						Name applied by Bardsley	No.
MR	VP	cit	UA	ind	gel		
+	-	-	-	+	-	B. coli	2947
+	-	-	-	-	-	Irregular	80
+	-	-	+	-	-	Irregular	3
+	-	+	-	-	-	Intermediate	581
+	-	+	+	-	-	Irregular	35
+	-	+	-	+	-	Irregular	16
+	-	+	+	+	-	Irregular	32
-	+	+	+	-	-	B. lactis aerogenes	478
-	+	+	+	+	-	B. lactis aerogenes	78
-	+	+	-	-	-	Irregular	36
-	+	-	+	-	-	Irregular	4
-	+	-	-	-	-	Irregular	7
.	.	.	.	.	+		12
+	+	.	.	.	.	Irregular	22
-	-	.	.	.	.	Irregular	2
						Total	4333

TABLE 36.

Correlations observed between the MR, VP, uric acid, and citrate tests.

(Bardsley, 1934)

Type	No.	Uric acid test				Citrate test			
		-		+		-		+	
		No.	%	No.	%	No.	%	No.	%
MR-VP+	603	43	7·1	560	92·9	11	1·8	592	98·2
MR+VP-	3694	3624	98·1	70	1·9	3030	82·0	664	18·0

TABLE 37.

Correlations observed between the MR, VP, uric acid, citrate and indole reactions.

(Bardsley, 1934)

Reactions:				Indole-positive		Indole-negative		Total
MR	VP	UA	cit	No.	%	No.	%	
+	-	-	-	2947	97·3	80	2·7	3027
+	-	-	+	16	2·7	581	97·3	597

she placed too much emphasis upon it, and needlessly classified too many of the strains as Irregulars. If, for example, this test is disregarded, the strains fall more simply into the groups shown in Table 38.

Most of the strains, she observed, fermented lactose within 48 hours, but some took longer. She allowed

up to 10 days at 37°C. before discarding any cultures. She also gave the same time to the litmus milk cultures. Any strains which had not succeeded by this time were placed in a water bath at 100°C. for a few minutes, and clotting then invariably took place. On the basis of her own nomenclature the percentages of slow lactose-fermenters and of weak clotters may be summarised as in Table 39.

TABLE 38.

Coliform types found in water (after Bardsley, 1934)

Reactions					Type according to Ministry of Health (1939)	No.	%
MR	VP	cit	ind	gel			
+	-	-	+	-	Bact. coli, type I	2947	68·0
+	-	-	-	-	Bact. coli, type II	83	1·9
+	-	+	-	-	Intermediate, type I	616	14·2
+	-	+	+	-	Intermediate, type II	48	1·1
-	+	+	-	-	Bact. aerogenes, type I	514	11·9
-	+	+	+	-	Bact. aerogenes, type II	78	1·8
-	+	-	-	-	Irregular	11	
+	+	.	.	.	Irregular	22	0·8
-	-	.	.	.	Irregular	2	
.	.	.	.	+	(? Bact. cloacae)	12	0·3
					Total	4333	100·0

Table 39.

Percentages of types (Bardsley) showing slow lactose fermentation and weak clotting.

(After Bardsley, 1934)

Type	Total No.	Strains giving AG in lactose				Strains clotting weakly
		48 hours		2-10 days		
		No.	%	No.	%	%
B. coli	2947	2934	99·56	13	0·44	2·37
Intermediate	581	309	53·18	272	46·82	41·65
B. l. aerog. (ind±)	556	437	78·59	119	21·41	22·66
Irregulars	213	158	74·17	55	25·83	26·30
Total	4297	3838	89·33	459	10·68	11·51

In an endeavour to correlate the type of coliform organism with its source, she classified the 2144 water samples into 4 groups:

(a) Town supplies - mainly upland surface waters.
(b) Rural supplies - mainly shallow wells and springs.
(c) Swimming bath waters - all indoor and chemically treated.
(d) Miscellaneous - ponds, streams, and other polluted sources

Her results are shown in Table 40.

TABLE 40.

Proportion of coliform types found in various kinds of water (Bardsley, 1934)

Source	Total No. of strains	B. coli (UA- cit-) %	Intermediate (UA- cit+ ind-) %	B. lactis aerogenes (UA+ cit+ ind±) %	Irregular strains %
Upland surface waters	2572	78·1	11·9	7·5	3·5
Shallow wells, springs	1375	55·4	18·7	18·6	7·3
Swimming bath waters	231	41·1	15·2	35·1	8·6
Miscellaneous (polluted)	119	68·9	8·4	21·0	1·7
Totals	4297	68·6	13·5	12·9	5·0

She noted the high percentage of coli occurring in the upland surface water, and the lower percentages in the other sources. The higher percentage of the citrate-utilisers in the shallow wells correlated with the likelihood of pollution by soil washings. The relatively great proportion of aerogenes, however, in the swimming bath waters could not be explained on this basis as whatever pollution occurred came direct from the bathers. Bardsley suggested that aerogenes, being more resistant to other adverse circumstances, might tolerate chlorine better than coli. As confirmation of this theory she pointed out that whereas in the other water groups coli was often isolated alone, in the case of the swimming bath waters the other types were more often encountered first (i.e., in the smallest volumes) and to find coli it became necessary to plate out all the positive tubes. In other words, the coliform flora of the swimming bath water was much more varied.

As a test of the accuracy of the presumptive test, she compared the number of samples which gave a positive presumptive result with the number yielding B. coli (see Table 41).

TABLE 41.

Source	Total No. of samples	Samples giving a positive presumptive result.		Positive samples yielding B. coli	
		No.	%	No.	%
Upland surface ws.	1622	750	46·2	647	86·3
Shallow wells, etc.	394	286	71·7	229	80·1
Swimming baths	90	37	41·1	30	81·1
Miscellaneous	33	29	87·9	28	96·6
Totals	2144	1102	51·8	934	84·7

She noted that in all groups at least 80% of the

samples giving a positive presumptive result yielded B. coli, and added that if the presumptive test had been considered sufficient evidence of B. coli only 15·3% of the positive samples would have been wrongly included.

As her investigation had extended over a period of 6 years she attempted to trace a seasonal incidence of coliform organisms and types in the water supplies, and noted that more samples were positive in summer than in winter. She found B. coli to show a definite increase in the summer and early autumn, during which period the other types became relatively less numerous, whereas in the late winter and spring months, B. coli - while still remaining the dominant organism - suffered some diminution and the other types increased correspondingly.

The soil samples, 86 in number, were collected with aseptic precautions from the Pennine moorlands in and near the Peak District, i.e., where the chances of faecal pollution would be extremely slight. Many of the samples were taken from waterworks' ground far removed from houses and farms, and unpolluted by grazing sheep. The possibility of contamination by birds, etc., could not, of course, be excluded.

A 1 in 10 dilution of the sample in sterile water was examined exactly as the water samples, but a high proportion of false presumptive reactions was obtained, due to the growth of B. welchii. Of the total 86 samples 65 (75·6%) yielded no coliform bacteria. From the remaining 21 soils were isolated 152 coliform strains, none of which were Irregular. The results are shown in Table 42.

TABLE 42.

Proportions of coliform types obtained from soil.

(Bardsley, 1934)

Type of organism		No.	%
B. coli	(MR+VP-ind+UA-cit-)	47	31·0
Intermediate	(MR+VP-ind-UA-cit+)	101	66·4
B. lactis aerogenes	(MR-VP+ind±UA+cit+)	4	2·6

TABLE 43.

Distribution of strains among the total 86 soil samples

(Bardsley, 1934)

Samples giving:	No.	%
No coliform organism	65	75·6
B. coli	7	8·1
Intermediate	17	19·8
B. lactis aerogenes	2	2·3

89 of the 101 Intermediate strains and 1 of the B. coli were slow lactose-fermenters but all 4 aerogenes

bacilli fermented lactose vigorously. The same morphological differences between coli and the Intermediates * were again observed. The distribution of the organisms among the soil samples was tabulated (see Table 43).

In view of the large proportion (over 75%) of the soils from which no coliform bacteria were recovered and which were, therefore, definitely free from faecal pollution, the remaining 21 soil samples, Bardsley considered, must be assumed to have been subject to some such pollution although true coli occurred relatively seldom. She noted the predominance of the Intermediate type in the soils she had examined, but, having studied the findings of previous investigators, decided that so much of the testimony was conflicting that without further observations it was impossible to infer more than that coliform bacilli did occur in soil, the relative proportions of the types varying in different areas.

Her examination of faeces extended to 34 specimens (submitted for investigation for pathogenic organisms) which were emulsified in peptone water and either spread direct on MacConkey agar or (in a few cases) given a preliminary incubation in citrate medium. Altogether, 331 coliform strains were isolated and tested, with the results shown in Table 44.

TABLE 44.

Proportion of coliform types in faeces.

(Bardsley, 1934)

Method of testing	No. of samples	No. of strains isolated	B. coli		Intermediate		B. lactis aerogenes		Irregular	
			No.	%	No.	%	No.	%	No.	%
Direct plating	32	220	194	88	17	8	6	3	3	1
Citrate	7	111	63	57	13	12	34	31	1	1
Totals	34	331	257	78	30	9	40	12	4	1

She noted that B. coli was the predominant organism in both series but drew attention to the advantage given to the citrate-utilisers, and particularly to aerogenes, by the citrate medium, in which aerogenes (she pointed out) must have grown vigorously. As before, she also studied the distribution of the types in the various specimens (see Table 45.)

TABLE 45.

Distribution of coliform types in faecal specimens.

(Bardsley, 1934)

Method of testing	Total samples examined	Samples giving							
		B. coli		Intermediate		Aerogenes		Irregular	
		No.	%	No.	%	No.	%	No.	%
Direct plating	32	30	94	5	16	3	9	2	6
Citrate	7	7	100	2	29	4	57	1	14

[*These were shorter and thicker than B. coli]

From her results she concluded that, while B. coli is definitely the dominant coliform organism in faeces, the Intermediate and aerogenes types also occur therein although in much smaller numbers.

The ice-cream samples numbered 44, 17 of which were found to contain no coliform organisms (in the volumes examined). The remaining 27 samples yielded 365 coliform strains classified as in Table 46.

TABLE 46.

Proportion of coliform types in ice-cream.

(Bardsley, 1934)

Type	No.	%
B. coli	26	7
Intermediate	70	19
B. lactis aerogenes	244	67
Irregular	25	7
Total	365	100

Bardsley noted the relatively small numbers of B. coli and the predominance of B. lactis aerogenes. She advanced two possible explanations: (1) that aerogenes types (MR-) were generally fairly common in milk and milk products such as were used for the making of ice-cream, and (2) that prolonged exposure to low temperature in the ice-cream-processing might destroy B. coli but allow aerogenes to retain its viability. In this latter connection she recalled her water results where the highest proportion of aerogenes occurred in the late winter and spring months, and suggested that survival at low temperature might be a character of aerogenes which could be collated with its apparent ability to withstand weak chlorine in swimming baths.

In her general conclusions Bardsley decided that "organisms of the coliform group are not widely distributed in nature except where faecal contamination has taken place at some period more or less remote." She recollected that most of the virgin soils examined and many of the upland surface waters had been free from coliform bacilli. Faeces contained enormous numbers of coliforms of which B. coli was the dominant type, but the presence of small numbers of the other types could be demonstrated by special methods of isolation. She concluded, therefore, that the presence of these types in food or water might be due to faecal pollution, though the absence of B. coli would suggest that the pollution had not been recent.

As regards the routine bacteriological examination of water, since the presumptive reaction was so closely associated with the presence of B. coli itself, and especially since the other coliform types possessed some

faecal significance, she felt that the differential tests were hardly justified and might well be discarded.

She summed up her findings and concluded that the relative proportions of the various types of coliform bacteria in food and water depended upon external conditions, and the relative proportions in soil seemed to be governed by local circumstances.

Summary and Discussion
(1920-1934)

The general interest now centred on the coliform group is reflected in the very large number of contributions made to the literature during this period. The classification was still in a state of flux. As regards the group itself, the vigorous lactose-fermenters were certainly included, but these gradually merged via slow-lactose-fermenters, non-gas-producers (anaerogenes) into the definitely non-lactose-fermenting glucose-fermenters. Mackie (1921) preferred to regard them all as coliform bacteria, but most bacteriologists tended to include only the definite lactose-fermenters. In this latter case, however, it became a matter of opinion as to what time should be allowed for the production of acid and gas. While for most of the lactose-fermenters a period of 24-48 hours was amply sufficient, some required 3 days, and a few even longer. These slow-lactose-fermenters were shown (Dudgeon, 1924) to be at least as capable as B. coli itself of causing urinary infections, but what exact sanitary significance they possessed was open to doubt. Despite the opinion of Wilson (1928) that they were of little sanitary importance, Bardsley (1934) excluded from her significant coliform strains only those organisms which failed to produce acid and gas in 10 days, and thus included in her water series nearly 11%, and in her soil series some 60%, of slow-lactose-fermenters. Some general agreement was needed on this subject.

In connection with the internal classification, or differentiation, the aim was to establish sub-groups which correlated with habitat and a precise sanitary implication. Except, therefore, for the efforts of systematists such as Bergey (1934) little attention was paid to the fermentation reactions, while the value of the MR and VP reactions was undergoing critical investigation. That these tests afforded a useful distinction was already universally agreed. The MR- types formed only a very small percentage of the coliform bacteria in faeces but were the predominant coliforms in soil and vegetation, constituting 95% of the strains in grasses and oats (Rogers et al, 1914), and 72% (Johnson, 1916), 81% (Burton & Rettger, 1917) and 96% (Chen & Rettger, 1920) of the coliforms found in soil. They could therefore be regarded almost as "non-faecal" organisms and

their presence in, for example, water need convey no (Bardsley, 1926) indication of faecal pollution, but merely the suggestion of soil washings.

Where, therefore, the MR- organisms were the predominant type the sanitary conclusion was easy, but further investigations revealed that they were not always in the majority in apparently innocent sites. Though Koser (1926) and Raghavachari (1926) continued to find percentages from 64 to 93 in soil, Burke-Gaffney (1932) obtained a figure of 76% in African soil and water, while Tonney and Noble (1931) established an aerogenes/coli ratio of 20:1 in soil and vegetation, Hicks (1927), on the other hand, recovered only 20% of MR- coliforms from Shanghai soils, and Bardsley (1934) obtained in England the extremely low figure of 2·6% aerogenes in soil. In other words, the MR+ organisms, which were always chiefly in evidence in faeces and faecally polluted sites, were sometimes similarly numerous in sites judged to be not so contaminated.

It was suspected - especially in the Tropics - that MR+ strains might be derived from soil as well as from faeces but until 1923 there was no way of distinguishing between these two varieties unless by means of the indole test of which sufficient use does not appear to have been made. The appearance of the citrate test - the great discovery of the period - supplied a long felt want, for it clearly differentiated the soil from the faecal strains. In other words, citrate-positive coliforms invariably predominated in sites from which faecal contamination could by other means be excluded (see Table 47.).

TABLE 47.

The prevalence of citrate-positive coliforms in relatively unpolluted situations.

Reference	Percentage of citrate+ coliform bacteria	Source
Koser, 1924	83 97	Unpolluted water Soil
Pawan, 1925	81 90	Unpolluted water Unpolluted soil
Koser, 1926 1926c	82 77	Soil Unpolluted soil
Raghavachari, 1926	95	Soil
Hicks, 1927	80	Soil
Burke-Gaffney, 1932	88	"Non-faecal" sources
Koser & Saunders, 1932	100	Soil

Thus, not only were the MR- types shown to be citrate-positive but most of the soil MR+ strains possessed

this characteristic while the faecal MR+ strains did not.

Hicks (1927) who had, perhaps, suffered most from the failure of the MR and VP reactions hailed this new test as the sine qua non, and suggesting discarding the MR.VP distinction for a citrate-indole combination. The position was well summed up by Wilson (1928):-

(a) MR+VP-citrate-, recent faecal pollution indicated;
(b) MR+VP-citrate+, does not indicate recent faecal pollution.
(c) MR-VP+citrate+, certainly does not indicate recent faecal pollution.

It was widely noted that the first group (coli) was practically always indole-positive, while the second (Intermediate) was nearly always indole-negative. Had this been as fully appreciated before 1923 much of the confusion might have been avoided. Nevertheless, the stability of the capacity to utilise citrate having been proved, the test was universally accepted for the sanitary differentiation of the coliform group, with particular success in the tropical countries where it was most needed. It was now apparent that in those non-polluted sites where aerogenes was not predominant, the gap was filled by the Intermediate sub-group.

As this period was so full of small detail, much has received comment in the course of the description. It remains only to point out that whereas Bardsley denied any faecal importance to the MR- types in 1926, she retracted from this position as the result of further study. Thus in 1934, while allowing that differentiation had its value in the hot countries, she had come to doubt whether it was necessary in England. This for 2 reasons: (1) the close correlation between the presumptive reaction and the presence of B. coli (80% upwards), and (2) the complete absence of all coliform types from sites which could be reliably pronounced unpolluted. This will be discussed more fully later, but here let me say that the presumptive reaction may be 80% right in the total of a series of examinations but at the same time 100% wrong in any particular one. The submitter of a sample is interested only in his own results and is entitled to proof, not supposition, where the existence of pollution of a serious nature is claimed. The views of Wilson (1928) are preferable: the quantitative test is the most important, but in certain cases a qualitative examination will help in the interpretation.

The Characters and Distribution of the Coliform Bacteria.

HISTORY AND LITERATURE

Period 1934 - 1944

The lack of uniformity in the technique employed by different bacteriologists in the routine examination of water caused the English Ministry of Health to issue in 1934 a pamphlet which laid down standard procedures for the bacteriological examination of water supplies, and which stressed, among the vast body of literature, those findings which had been proved to be reliable. On the technical side, a limit of 48 hours was set for the presumptive test, and a differential classification was made on the basis of the MR, VP, citrate, indole and gelatin tests. This (I quote from memory because my copy was destroyed by enemy action) is indicated in Table 48.

TABLE 48.

Differentiation of the coliform group.

(Ministry of Health, 1934)

Sub-group	Cit.	MR	VP	Ind.	Gel.	Probable Habitat
Bact. coli, type I, faecal.	-	+	-	+	-	Human and animal intestine.
Bact. coli, type II	-	+	-	-	-	Doubtful
Intermediate, type I	+	+	-	-	-	Mainly soil.
Intermediate, type II	+	+	-	+	-	
Bact. aerogenes, type I	+	-	+	-	-	Mainly vegetation.
Bact. aerogenes, type II	+	-	+	+	-	
Bact. cloacae	+	-	+	-	+	
Irregulars	Reactions variable					Doubtful.

In this way, current proved knowledge was summarised, correlated and simplified. As this pamphlet was later revised (1939) further description will be postponed.

In an earlier section, mention was made of a high temperature fermentation test which had been introduced by Eijkman (1904). Eijkman (1904, 1914) showed that B. coli of faecal origin, but not B. lactis aerogenes, was able to grow in a glucose-broth medium at 46°C. and to ferment the carbohydrate with the production of acid and gas. For some reason (probably the unusual temperature of incubation) this test did not attract attention until nearly 1930 when a few workers independently started to study it. From that time onwards, the test gradually came into prominence, receiving favorable comment from some quarters, notably Perry (1929) and Levine, Epstein and Vaughn (1934), and the reverse from others, e.g., Ruchhoft et al., (1931), Burke-Gaffney (1932), and Webster and Raghavachari (1934). Perry and Hajna

(1935), having found large numbers of the intermediate-aerogenes-cloacae (I.A.C.) coliform types in unpolluted shellfish and having thereby explained the failure of the ordinary (37°C.) presumptive test to distinguish between serious and unimportant pollution of shell-fish, advocated the substitution of the presumptive test by Eijkman's test in shell-fish testing. They retained the original temperature (46°C.), allowing a mere bubble of gas to constitute a positive reaction, but replaced the Eijkman medium by MacConkey (lactose) broth. By this means they showed that out of 233 lactose-fermenters obtained from unpurified oysters only 11·2% were true coli.

Other workers, while confirming the specificity of the test for faecal coli, suggested that 44°C. gave better results, and the matter was thoroughly investigated by G.S.Wilson and his assistants (1935). They decided that 44°C. was the optimum temperature, 46°C. being too high to permit all the faecal coli to develop; they also preferred MacConkey's medium and stressed that the production of gas was the important criterion. For the test to be specific for faecal coli they found that it was essential for the temperature in the medium not to vary by more than ± 1°C., and for this reason they recommended a water-bath in preference to the best of incubators. Applying the test to coliform bacteria they had isolated from milk, cow-dung, feeding materials, etc., they showed that a positive result was practically specific for Bact. coli, type I, and obtained an almost complete inverse correlation with the citrate test. Out of 496 coliform strains only 1 gave a double positive result. Commenting on the importance of accurate and constant temperature, Wilson summed up the discordant previous reports thus: "It seems likely that those workers who have reported on the test favorably have had incubators permitting a fairly constant temperature of about 44°C. in their Eijkman tubes, while those who have reported on it unfavorably have been working with incubators in which the temperature of the medium was too high, too low, or inconstant." Referring, doubtless, to the somewhat unsatisfactory results obtained by Harold (1933-5) of the Metropolitan Water Board Laboratories, who had used 42°C., Wilson observed that this temperature permitted occasional strains of aerogenes to form gas. Harold nevertheless continued to use 42°C. until 1937. Wilson further proved that faecal coli could grow in MacConkey's medium at 44°C. as freely as at 37°C, and this property it shared with only a few Irregular strains.

Using this test as an additional criterion, Wilson elaborated the Ministry classification as shown in Table 49. In the final column appear the numbers of each type isolated from milk, etc. Wilson's comments upon the most probable significance of the different types are set out in Table 50.

TABLE 49.

Classification of coliform strains.

(Wilson et al., 1935)

Type	MR	VP	Cit	Ind	44°C	Gel	No. of strains
Bact. coli, type I	+	-	-	+	+	-	180
Bact. coli, type II	+	-	-	-	-	-	39
Intermediate, type I	+	-	+	-	-	-	87
Intermediate, type II	+	-	+	+	-	-	10
Bact. aerogenes, type I	-	+	+	-	-	-	99
Bact. aerogenes, type II	-	+	+	+	-	-	14
Bact. cloacae	-	+	+	-	-	+	31
Irregular I, coli-like 1	+	-	-	+	-	-	13
Irregular II, coli-like 2	+	-	-	-	+	-	9
Irregular III, coli-like 3	+	-	-	-	-	+	1
Irregular IV, intermediate-like	+	-	+	-	-	+	5
Irregular V, aerogenes-like 1	-	+	-	-	-	-	4
Irregular VI, aerogenes-like 2	-	+	+	-	+	-	1
Irregular VII	-	-	+	+	-	-	2
Irregular VIII	-	-	-	-	-	-	1
Total							496

Table 50.

Most Probable Significance of the Coliform Types Isolated.

(Wilson et al., 1935)

Type	Habitat
Coli I	Far and away the commonest organism in human and bovine faeces.
Coli II	Infrequent in human and bovine faeces, quite common in raw milk, hay and straw. Origin uncertain.
Intermediate I	Commonest type in hay, not found at all in cow-dung. No evidence to show it to be indicative of excretal pollution. Origin ? soil.
Intermediate II	Found in raw milk. Origin unknown.
Aerogenes I	Commonest coliform in raw milk and grains. Small numbers in cow-dung.
Aerogenes II	Found in milk but not in cow-dung.
Cloacae	Found in milk and food-stuffs, but not in cow-dung.
Irregular I & II	Origin probably intestine.
Irregular III	Very uncommon anywhere.
Irregular IV	Found in grains and milk.
Irregular V & VI	Found only in milk.
Irregular VII & VIII	Found in milk and food-stuffs.

With the exception of Irregulars I and II, which constitute a very small minority, Bact. coli, type I,

was therefore the only type which gave certain indication of faecal contamination. The positive 44°C. results thus correlated absolutely with faecal significance. That the 44°C. test was practically specific for faecal coli was subsequently confirmed by Bardsley (1938) working with faeces, Dodgson (1938) in connection with shell-fish, Mackenzie and Hilton-Sergeant (1938), and Clegg and Sherwood (1939).

G.S.Wilson et al. (1935) also tested each of the 496 strains for motility, encapsulation, colony characters, and fermentation of various sugars. Some of their results are shown in Table 51.

Table 51.

Some characters of coliform types (Wilson, 1935)

Strains	Percentages		
	Motile	Capsulated	Mucoid Colonies
Bact. coli	70	20	20
Intermediate	44	25	51
Bact. aerogenes	47	67	73
Bact. cloacae	84	48	61

"It was rather surprising," they state, "to find that quite a large number of capsulated strains from mucoid colonies were actively motile in young cultures."

Tittsler and Sandholzer (1935) made a special study of the Intermediate coliform types. These, they found, varied with regard to citrate, H_2S, indole, cellobiose, and methyl glucoside. They concluded that the Intermediate coliform types were so heterogeneous in character that their inclusion in a separate genus was hardly justified.

Sherman (1935) proved that small numbers of the aerogenes types were constantly present in animal faeces, and Platt (1935) confirmed the greater viability of the aerogenes types in stored water. Malcolm (1935) concluded that a positive citrate result was an important index of viability; thus, citrate-positive types were, he found, more resistant to brilliant green.

While agreeing that the presumptive coliform reaction was in Great Britain a reliable indication of the presence of coliform bacteria, Atkinson and Wood (1938) contended that in other countries, where the water flora was different, false presumptive reactions might not infrequently be encountered.

Harold (1937) noted that in waters which were very slightly polluted and contained only very small numbers of coliform bacteria the I.A.C. types were often more numerous than faecal coli. Bardsley (1938) confirmed this finding. She also examined 100 specimens of faeces

of faeces by a selective method and found coli I present in every specimen but I.A.C. organisms only in 61. Bact. coli was the dominant organism in 92 specimens; in 2 it was present in numbers equal with those of the I.A.C., and in 6 it was numerically inferior to the I.A.C. types.

In 1939 the Ministry of Health issued a revised edition of their 1934 Report. They now advocated the 44°C. test (± 0·5°C.) as a useful confirmatory test for faecal coli, emphasising that its specificity had been established in most countries. They adopted the test in a revised scheme of classification, modified from that of Wilson (1935), which is reproduced in Table 52.

TABLE 52.

Differentiation of the coliform group.

(Ministry of Health, 1939)

Type	MR	VP	Cit	Ind	Gas at 44°C.	Gel	Probable habitat.
Bact. coli, type I, faecal.	+	-	-	+	+	-	Human and animal intestine.
Bact. coli, type II	+	-	-	-	-	-	Doubtful; probably not primarily intestinal.
Intermediate, type I	+	-	+	-	-	-	Mainly soil.
Intermediate, type II	+	-	+	+	-	-	
Bact. aerogenes, type I	-	+	+	-	-	-	Mainly vegetation.
Bact. aerogenes, type II	-	+	+	+	-	-	
Bact. cloacae	-	+	+	-	-	+	
Irregular, type I	+	-	-	+	-	-	Human and animal intestine.
Irregular, type II	+	-	-	-	+	-	Doubtful.
Irregular, other types	Reactions variable						Doubtful.

Stressing the faecal importance of Bact. coli, type I, they noted that is was "far and away the commonest type of coliform organism present in human and animal" faeces, and that "apart from excretal contamination it is rarely found outside the animal body." They stated that the I.A.C. group, though often found in small numbers in the intestinal canal, appeared to have their primary habitat in soil and on vegetation.

As regards the examination of water they expressed their preference for frequent simple examinations to less frequent but more elaborate ones, and built up a set of standards based on the presumptive tests alone. This will be discussed later.

Clegg and Sherwood (1939) elaborated a special thermo-regulator which reduced the temperature variation in their 44°C. bath to ± 0·1°C. By experimenting over a

temperature range of 41-46°C. they proved that 44°C. was definitely the best temperature for distinguishing the faecal coli (Bact. coli, type I) from the other coliform types. They noted the existence of 'border-line' strains, however, which required confirmation (or the reverse) by the other differential tests, but found that most of the cultures which fermented lactose at 44°C. did so vigorously, giving a positive result within 24 hours.

Workers in tropical countries did not obtain such good results with this test. Webster (1934-5) in India reported on it unfavorably, but he had used a temperature of 46°C. and this, doubtless, contributed to his lack of success. Using 44°C., Raghavachari and Iyer (1938-9) reported similar dissatisfaction, however, and showed that about 50% of the I.A.C. group which they had isolated from waters in India could give gas at 44°C., either after direct inoculation or after preliminary incubation at 37°C. Boizot (1941) proved that the test was not so specific in Singapore as in the more temperate regions, though his results were much more encouraging than those of the Indian workers. He concluded that, while results must be cautiously interpreted, the test was definitely helpful in Singapore, where the waters contained relatively large numbers of the I.A.C. types.

Though this test must be considered less valuable in the tropics (where the need for it is greatest), it has continued to receive approbation from workers in this country and America. Ferramola (1940) has proved it to be equally efficacious in the Argentine. Mackenzie (1940) noted the direct application of the 44°C. test to waters revealed rather less faecal coli than preliminary incubation at 37°C. or 42°C. followed by plating, but this has not been the experience of more recent workers here (Sherwood and Clegg, 1942; Batty-Smith, 1942). Batty-Smith (1942) has made this test the subject of an exhaustive study. Having surveyed the literature and decided from this and his own results that it is the finest single test available for faecal coli, he shows that, used in place of the usual 37°C. presumptive test, it is often more sensitive for Bact. coli, type I, than the older methods, besides being more economical. He agrees, nevertheless, that it cannot replace or supplant the ordinary presumptive test because it gives no information on the presence or incidence of the other coliforms which it must be remembered (Suckling, 1943, 1944) are not devoid of sanitary significance.

A most notable contribution to the classification of the coliform bacteria was made in 1938 by Malcolm, who emulated MacConkey in the thoroughness of his study. Reporting the results of investigations he had made (1933, 1935) into the coliform flora of milk and bovine faeces, he (1938) aimed at improving upon the current

methods of classification. He noted that in routine sanitary examinations of water and milk no attempt was made to identify individual strains, yet as a dairy bacteriologist he felt that the recognition of such types might be of great importance in connection with disease conditions or the causation of specific taints in dairy produce. To show how controversial was the question of coliform classification, he alluded to the differences between the schemes of Mackie (1921), as endorsed by Muir and Ritchie (1937), and that of Bergey (1934); and, in reviewing the literature, he described MacConkey's classification as possessing the disadvantage of leading to a multiplicity of types "the differences between which are so slight as to be of little or no significance in public health or dairy advisory work and even for systematic purposes." In criticising Bergey's scheme of classification, Malcolm made the following points: (1) The coliform group of bacteria consists of a gradation of types so closely linked as to render it undesirable to divide the group into two genera. (2) The indole and inositol tests are not used as differential criteria despite their importance as shown by Mackie (1921), Hay (1932) and Malcolm (1933, 1935). (3) The gelatin reaction, on which Bergey places considerable reliance, is unsatisfactory as requiring too much time (up to 12 weeks). (4) Salicin, which Bergey uses for differentiating species of Escherichia, is unsuitable as, in the experience of Mackie (1921) and Malcolm, it subdivides even such classical strains as B. coli communis and B. coli communior. (5) The subdivision of the Aerobacter types on sucrose and dulcite is unsatisfactory as affording no correlation with indole-production which, Malcolm contends, is a more important criterion.

Summing up his study of the literature, Malcolm states: "In the various systems of classification which have been used, differences occur, not only in the criteria employed for differential purposes, but, when the same criteria have been adopted, in the value attached to them." Malcolm finally decided to use MacConkey's criteria together with the citrate test for the purpose of differentiating into individual strains, and then to adopt some system of re-grouping afterwards.

From milk and bovine faeces he isolated a total of 1636 coliform bacteria which had the following common characters: Gram-negative, non-sporing rods capable of growing aerobically at 37°C. and fermenting glucose and lactose with the production of acid and gas. Of the total, 797 organisms were obtained from milk and 459 from bovine faeces by ordinary methods of isolation. A further series of 380 strains was obtained from faeces by a citrate enrichment method similar to that of Gray (1932), in the course of which all "typical coli" cultures isolated were discarded. Each strain was tested for the common characters already mentioned and then

subjected to the following tests: motility, encapsulation, production of indole, citrate-utilisation, MR and VP reactions, liquefaction of gelatin, and fermentation of sucrose, dulcitol, adonitol, inositol, inulin, mannitol, raffinose, and salicin. The milk strains were grouped according to the citrate, indole, MR and gelatin reactions, and the faecal strains were similarly grouped except that inositol-fermentation was substituted for the gelatin test. Malcolm's time limits were generous: 7 days were allowed for the citrate test, 10 for indole-production, and 14 for the fermentation reactions, while 12 weeks' incubation at 37°C. was allowed for the gelatin test.

Except for 39 anomalous strains (approximately 2·5% of the total) a close degree of correlation was established in all cases between the citrate, indole, MR and inositol reactions, and, using these tests as his basis, Malcolm was able to place each of the remaining 1597 strains in one of 8 sub-groups. These, together with the number of organisms, are shown in Table 53.

TABLE 53.

Classification of the coliform bacteria and the numbers isolated from milk and bovine faeces

(Malcolm, 1938)

Sub-group	Tests				Ordinary methods		Enrichmt. methods	Total
	VP	Cit	Inos	Ind	Milk	Faeces	Faeces	
1. B. coli	-	-	-	+	435	447	(discarded)	882
2. Intermediate	-	+	-	+	1	0	17	18
3. Intermediate	-	+	-	-	74	2	50	126
4. Intermediate	-	+	+	+	13	0	10	23
5. Friedlander	-	+	+	-	20	1	12	33
6. B. cloacae	+	+	-	-	94	2	115	211
7. B. oxytocus	+	+	+	+	22	3	46	71
8. B. aerogenes	+	+	+	-	105	4	124	233
Totals					764	459	374	1597

TABLE 54.

Characters of the types isolated (Malcolm 1938)

Subgroup	Motility		Adonitol		Sucrose		Dulcitol		Inulin		Gelatin		Total
	+	-	+	-	+	-	+	-	+	-	+	-	
1.	785	97	106	776	512	370	692	190	0	882	5	877	882
2.	13	5	7	11	12	6	12	6	0	18	1	17	18
3.	124	2	1	125	48	78	40	86	0	126	9	117	126
4.	1	22	23	0	23	0	7	16	1	22	0	23	23
5.	4	29	33	0	33	0	14	19	5	28	3	30	33
6.	198	13	4	207	211	0	59	152	13	198	141	70	211
7.	4	67	70	1	71	0	55	16	59	12	47	24	71
8.	34	199	222	11	232	1	42	191	43	190	24	209	233

The degree of correlation between each subgroup and the fermentation, etc., reactions is shown in Table 54, from which it is evident that these, as a whole, do not afford more than statistical help in classification. Thus, except for the facts that the first three subgroups do not ferment inulin and that the last five subgroups ferment sucrose, there are no absolute associations, though the balance is often heavily weighted on one side or the other. While allowing that the characters are not invariably adhered to, it is possible nevertheless to work out the common or typical reactions for each subgroup (on a majority basis). Malcolm's remarks in this connection are summarised in Table 55.

TABLE 55.
Characteristic reactions of coliform subgroups.
(Malcolm, 1938)

Subgroup	Citrate	VP	Inositol	Indole	Motility	Encapsulation	Mucoid colonies	Gelatin liquefn.	Adonitol	Inulin	Sucrose	Dulcitol	Habitat
1. B. coli	-	-	-	+	+	-	-	-	-	-	±	±	Faeces
2. Intermediate	+	-	-	+	+	-	-	-	+	-	±	±	Small nos. in soil & faeces
3. Intermediate	+	-	-	-	+	-	-	-	-	-	+	+	Soil. Small nos. in faeces
4. Intermediate	+	-	+	+	-	+	+	-	+	-	+	+	Seldom in soil or faeces
5. B. friedlanderi	+	-	+	-	-	+	+	-	+	-	+	+	Small nos. in faeces
6. B. cloacae	+	+	-	-	+	+	+	±	-	-	+	+	Soil, plants & faeces
7. B. oxytocus	+	+	+	+	-	+	+	±	+	+	+	±	Small nos. in faeces
8. B. aerogenes	+	+	+	-	-	+	+	-	+	±	+	+	Soil, plants & faeces

Mannitol was fermented by all the strains and was excluded from the tables. The MR reaction was also omitted as it gave a perfect inverse correlation with the VP reaction. Raffinose and salicin were disregarded, the former because it correlated with sucrose and the latter as being of no value. There seems to have been little advantage, in this respect, in including dulcitol.

The 39 "anomalous" strains, most of which had been obtained from milk, had the characters shown in Table 56.

In Table 57 are rearranged, according to the Ministry classification, the 459 strains isolated by ordinary methods from cow faeces. It is noteworthy that Bact. coli, type II, is not present, and from the context none of the "anomalous" strains belonged to this series. It would seem, therefore, that, in cow faeces at least,

coli II is even more infrequent than the citrate-utilisers.

TABLE 56.

Reactions of the "anomalous" strains.

(Malcolm, 1938)

No. of strains	VP	Cit.	Inos.	Ind.	Ministry type
7	-	-	-	-	Bact. coli, type II
7	+	-	-	-	Irregulars
3	+	-	+	+	
3	+	-	+	-	
19	+	+	-	+	Bact. aerog., type II

TABLE 57.

Coliform types found in bovine faeces.

(Malcolm, 1938)

Ministry type	No.	%
Bact. coli, type I	447	97
Bact. coli, type II	0	3
Intermediate, type I	3	
Intermediate, type II	0	
Bact. aerog., type I	4	
Bact. aerog., type II	3	
Bact. cloacae	2	
Totals	459	100

Malcolm draws attention to the occasional occurrence of ill-defined reactions, and, in justification of his classification scheme, notes that "border-line strains, i.e., those which appear to belong to recognized types but are atypical with regard to one or other of the subgroup criteria, present a problem in the classification of coliform bacteria, no matter what criteria are used."

In discussing his findings, Malcolm confirms the general decision that his subgroup No. 1 (typical B. coli, Bact. coli, type I) possesses special importance, in that it includes those types which are by far the most prevalent in human and animal faeces, comprising the great majority of coliform cultures obtained from such sources when isolations are made by the usual methods. "Organisms of these types may also be found in soil, in water and on plants; but in absence of recent faecal contamination their incidence in such environment is comparatively low and in many instances they are not to be found. It would appear therefore that they thrive best in the intestines and cannot persist indefinitely in soil and water."

With regard to the so-called "non-faecal" types, he

endorses the views of Bardsley (1934) in that they are almost always present in small numbers in human and animal faeces, as can be well demonstrated by enrichment methods of isolation; and, that, though they may be found in soil and water and on grains, even in the absence of recent faecal contamination, they are apparently not widely distributed in nature except where there has been, at some time or other, such contamination. He notes that they are the dominant type in soil and are evidently better adapted than B. coli to live in a natural environment.

"Having regard to the foregoing," he concludes, "it is evident that the incidence of any type of coliform organism in soil or in water serves as an indication of faecal contamination. The presence of large numbers of subgroup No. 1 (B. coli types) in water is evidence of recent sewage pollution. On the other hand, if organisms of only subgroups 2-8, e.g. B. aerogenes and B. cloacae types, are present, there still remains the probability that faecal contamination has occurred, but not at a recent date."

The recent work of Parr (1936, 1937, 1938, 1939) is of considerable interest. He studied the coliform flora of a large number of specimens of human faeces, and confirmed the general opinion that B. coli predominated. He found, however, that considerable variations from the usual proportions occurred in healthy persons, and that, even in the same individual, the flora could vary greatly from day to day. The extreme examples were one subject, in whose faeces on two successive occasions no coliform bacteria could be found, and another subject whose faeces showed the presence only of Intermediate, type I.

Experimenting with stored faeces, Parr (1936) kept specimens in saline suspensions in the ice-box, and noted a considerable change in the flora. Coli, at first dominant, gradually decreased so that the proportion of the I.A.C. types became relatively greater. Finally, after many months, there was a complete change in the flora, the only surviving coliforms being slow lactose-fermenting varieties of citrate-utilisers. In 14% of cases, however, these changes did not occur, Bact. coli persisting for months. The probable explanation for this, Parr suggests, is that in these cases the coliform flora was a pure culture of B. coli to begin with. "The significance of these findings," he states (1939), "is that all of the coliform bacteria must be thought of as possibly faecal in origin. Where pollution derives from several sources, one may expect to find E. coli if the pollution be fresh; where pollution is from a single source there is no certainty that E. coli will be present; and the finding of E. coli may not indicate fresh pollution if that pollution be derived from a single source. Despite these qualifications, the presence of significant numbers

of E. coli in water remains our best test for faecal pollution."

"Coliform bacteria," he continues later, " do not occur ordinarily in water except from contamination with soil washings and faecal material from man and animals. When the pollution is from faeces these bacteria survive for some time but generally with a shift from citrate-negative predominance to citrate-positive predominance. There are, however, theoretical and actual conditions under which E. coli may persist with typical reactions for long periods of time. Usually, though, the numbers of coliform bacteria decrease and, in the absence of re-contamination, the group is usually lost sight of after a few weeks."

Parr points out that the citrate reaction is not altogether a permanent characteristic. He notes that Minkewitsch et al.(1936) had reported the change of a definite E. coli, which they had seeded in soil, into a citrate-utilising Intermediate (type II). Parr had (1938) himself kept one of Koser's original soil strains of Intermediate (type II) in laboratory culture for over 10 years and derived E. coli. Of the coliform flora of soil, Parr states: "Despite the fact that citrate-utilisers predominate there is evidence that E. coli can survive for a considerable time in soil."

Parr shares the views of Mackie (1921) in respect of those organisms which should be included within the coliform group. Apart from the strict types which are universally recognized as coliform bacteria, Parr would include the following classes as atypical coliforms:

(1) those lactose-fermenters which differ from E. coli as regards the presence of capsules, liquefaction of gelatin; the MR+VP+, MR-VP- types, etc.

(2) slow lactose-fermenters (many of which are B. coli mutabilis - see later).

(3) the anaerogenes type ("non-gas-producers" of Mackie).

(4) the paracolon types.

Discussing variation in the coliform group, he recalls the original description by Massini (1907) of B. coli mutabilis, the classical example of bacterial variation. This organism, if grown on MacConkey agar, first develops a colourless colony in the centre of which, after 3 days, a red papilla appears. Subculture of the central papilla produces ordinary lactose-fermenters, but subculture of the pale peripheral part of the colony results in further changes of the type just described. This variation occurs with other sugars in certain cases, e.g., sucrose and dulcite. Stewart (1926) showed that B coli mutabilis, B. friedländeri, B. coli, and B. paracoli were closely related; that they were, in fact,

"Mendelian variants of one species. Mutabile is the central heterozygote, paracolon the pure dominant, and colon the pure recessive."

Parr notes, however, that variation in the coliform group is not confined to this phenomenon. Apart from the examples already mentioned in connection with citrate-utilisation, which he admits are uncommon, he points out that Sherman and Wing (1937) obtained coliform strains (respectively fermenters of salicin and sucrose) among whose descendents were non-fermenting varieties, and vice versa. He also affirms to have witnessed changes from MR+VP- to MR-VP+.

As regards B. coli anaerogenes, he quotes Kline (1930) as finding 126 anaerogenic E. coli among 325 cultures from raw and pasteurised milk, and believing that these were really coliform bacteria modified by unfavorable environment.

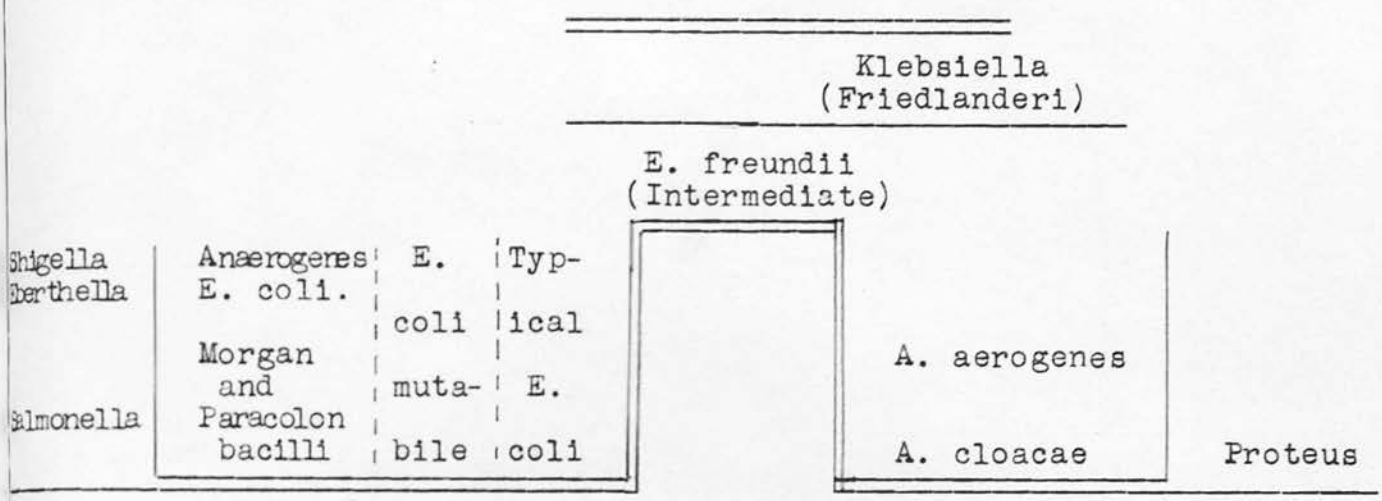
"We believe," continues Parr, "the evidence warrants the view that the slow lactose-fermenters, the anaerogenes strains, Morgan's bacillus and paracoli strains are all coliform bacteria which may be placed with whichever species they have the most characters in common." He notes that Minkewitsch et al.(1936), as the result of their experiments, suggested that all coliform bacteria might arise from faecal coli. "Most workers, however," says Parr, "seem to feel that the direction of evolution in the coliform group has been from the highly reactive, ubiquitous A. aerogenes to the less reactive, more specialised parasitic types."

"The reviewer hopes to convey a concept of coliform bacteria as a group of closely related, closely intergrading, bacteria in which, by the dropping of one character or the acquisition of another, an organism appears as a new strain.

"The coliform group is a large one made up of closely related highly intergrading and somewhat unstable bacteria which form a fairly wide gamut or continuum extending from the lactose-negative paracolon at one extreme to the highly reactive A. aerogenes at the other. Standing with the paracolon forms next to Salmonella one finds the Morgan bacillus. In about the same position and leading to Eberthella and Shigella are located the anaerogenes E. coli. Next to these varieties come the slow lactose-fermenting E. coli so likely to be manifested as "unstable variants." Completing one side of the picture one finds the typical E. coli which bridges over to the A. aerogenes side by way of the "Intermediates". Below A. aerogenes we find Cloacae which appears to point towards the genus Proteus, and above all forms, but particularly above A. aerogenes are located the Friedlander organisms."

Accompanying this description is a diagram which is

reproduced herewith:



Though this idea must have been in the minds of many bacteriologists for long enough, I have never before seen it so beautifully expressed.

The degree of possible variation in the coliform group was thoroughly investigated by Griffin and Stuart (1940) who selected some 457 coliform strains (most of which were unusual types or displayed some anomaly) and found 116 of them to be unstable. As the result of previous studies (Stuart et al., 1938) in which they had used the indole, MR, VP, and citrate reactions together with fermentation of cellobiose, as differential tests, they had classified the coliform group into 19 types, 6 of the usual subgroups being each split into 3 according to whether the cellobiose test resulted in acid and gas, acid only, or a completely negative reaction. Of the total 116 "shifts" found, 42 were due to such relatively minor changes in the cellobiose test, but a number of major variations were also discovered (see Table 58). Unfortunately the conditions under which these shifts occurred are not stated, and some of the variations are admitted to have not been spontaneous. The authors noted that relatively few of the shifts involved a change from one group to another and that the indole reaction appeared to be relatively stable.

Griffin and Stuart (1940) also investigated the coliform flora of soil. They obtained 205 coliform strains from "recently contaminated" soil (pasture lands, barnyards and recently fertilised fields), 213 from "remotely contaminated" soil (fertilised $1\frac{1}{2}$-2 years prior to taking the samples) and 174 from "uncontaminated" soil (a reservoir watershed which had been under rigid control for more than 10 years). Table 59 shows the proportions of the types isolated.

TABLE 58.

Variation in the coliform group.

(Griffin and Stuart, 1940)

Type	Change	Result	Number	Reversible
Coli I	Loss of indole-production	Coli II	3	Yes
	Develt of citrate-utilisn	Inter II	7	Yes
Coli II	Develt of citrate-utilisn	Inter I	2	Yes
	Reversal of MR,VP reactions	Irreg.	4	Yes
Inter I	Develt of indole-production	Inter II	5	No
	Develt of VP+ reaction	Irreg.	6	Yes
Inter II	Develt of VP+ reaction	Irreg.	15	Yes
Aerog I	Reversal of MR,VP reactions	Inter I	8	No
	Develt of indole-production	Aerog II	3	Yes
	Develt of MR+ reaction	Irreg.	11	Yes
Aerog II	Reversal of MR,VP reactions	Inter II	2	No
Irreg.	Develt of citrate-utilisn	Aerog I	10	No

TABLE 59.

Proportions of coliform types found in soil.

(Griffin and Stuart, 1940)

Type of soil	No. of strains	Esch.		Inter.		Aerog.	
		No.	%	No.	%	No.	%
Recently contamd	205	71	35	7	3	127	62
Remotely contamd	213	19	9	21	10	173	81
Uncontaminated	174	4	2	8	5	162	93

Subjecting these findings to the chi-square test, they concluded that "the facts strongly suggest that Aerobacter and intermediates constitute the normal coliform flora of the soil, since their occurrence is statistically independent of faecal contamination."

Like Parr, they felt that the slow lactose-fermenters and the anaerogenes types should be included as atypical forms within the coliform group. They admitted, however, that they had found very few anaerogenes coliform types in faeces while the incidence of these forms in non-faecal sources appeared to be extremely high.

In their summary they contended that Aerobacter and intermediates constitute the normal flora of non-faecal sources, while Escherichia are normal to faeces. The occurrence of these groups outside their normal habitat, they therefore suggested, is adventitious. Most of the aberrant lactose-fermenters, they concluded, appear to be of questionable sanitary significance. The practice of distinguishing between aerogenes and cloacae was, they considered, of doubtful value.

The intermediates were the subject of a special study by Vaughn and Levine (1942). These types, they stated, had the following characters: generally motile, giving acid and gas in lactose, MR+, VP-, citrate+, generally producing H_2S, fermenting cellobiose, utilising urea but not uric acid, 44°C. negative. They did not consider the production of indole to be of significance for generic differentiation (though very useful for specific identification).

As regards the claim that intermediates are probably not normal intestinal inhabitants, they felt that such a conclusion is not warranted, and advanced the figures shown in Table 60.

TABLE 60.

Proportion of coliform types found in various sites

(Vaughn and Levine, 1942)

Source	No. of strains	Coli %	Inter %	Aerog %	Non-class. %
Faeces	5010	86·0	7·1	5·4	1·5
Unpolluted soil	527	10·3	11·9	75·9	1·9
Remotely polluted soil	340	26·2	21·2	50·9	1·7
Recently polluted soil	799	25·3	8·6	63·5	2·6
Unpolluted water	4209	34·2	14·7	45·0	6·1
Polluted water	2452	58·7	7·8	28·8	4·7

From these findings they concluded that the intermediates are widely distributed in nature, only small numbers being present in faeces, and decided that their sanitary significance was difficult to ascertain.

As regards the methods of examination, they stressed the importance of proper technique in the performance of the differential tests, especially the VP reaction, and expressed the need for incubating at 30°C. They noted that the intermediates varied in respect of the production of H_2S and proposed dividing the group into 2 genera:

(1) H_2S-producing: E. freundii,
(2) H_2S-non-producing: E. intermedium nov. comb.

Before passing to the recent comprehensive review of Taylor (1942), a few contributions on technique made during this period deserve brief mention. Raghavachari and Iyer (1934) advanced their opinion that MacConkey's liquid medium was still unequalled for the primary isolation of coliform bacteria. They reviewed the literature concerning the occurrence of false presumptive reactions and noted that the bile salt in MacConkey's medium reduced these to an almost negligible minimum. This is also discussed by Suckling (1943). By experiments, Webster and Raghavachari (1935) proved that, even in

India, an ice-boxed sample of water, examined after a delay of 24-48 hours, gave results identical to those obtained by making inoculations from the fresh sample on the site.

The delay associated with the original VP test led O'Meara (1931) to introduce an accelerating modification (the addition of a little creatine). This has been commended by many workers, including Wilson et al. (1935), Muir and Ritchie (1937), Mackie and McCartney (1942) and Suckling (1943). Barritt (1936) advised the addition of α-naphthol as having an intensifying effect whereby he could demonstrate the production of acetyl methyl carbinol by many organisms previously thought to be VP-negative. This modification, however, does not appear to have gained general acceptance.

In an endeavour to decide the source of the coliform bacteria present in milk, Rowlands (1939) studied the udder, and concluded that a healthy udder did not constitute a potential reservoir for the multiplication of coliform bacteria. Infection of the udder, he stated, was rapidly followed by the clinical signs of mastitis and by changes in the milk. Injury of the teats leading to malformed tissue might, however, cause infection of the milk with coliform organisms owing to the difficulty of thorough cleansing.

Taylor (1942), in a comprehensive review of the literature on the subject of the ecology and significance of the coliform bacteria found in water, notes the growing tendency to separate the coliform group into the Bact. coli (faecal) and Bact. aerogenes groups. In discussing the significance of the latter he points out that some authorities regard Bact. aerogenes as a normal inhabitant of the soil and do not associate it with faecal pollution, while others consider Bact. aerogenes to be an indicator of remote pollution, having noted the presence of small numbers in faeces and its greater powers of resistance. In connection with the former view he quotes Topley and Wilson (1931): "and there seems equally little doubt that the Bact. aerogenes-Bact. cloacae group so demarcated consists of bacilli which live normally on plants or in the soil and not in the intestines of man and animals." Also: Bact. aerogenes "is not a normal inhabitant of the intestine, but occurs on plants and grains and in the soil." To decide whether this view could be justified was the main purpose of his study of the literature.

He notes in passing that in his own examination of the coliform flora of the lakes and streams in Westmorland (Taylor, 1941) his results, contrary to expectation, showed a greater proportion of aerogenes types in the more polluted of the lakes examined. In this paper the

differences are striking (see Table 61).

TABLE 61.

Proportions of coliform types in waters.

(Taylor, 1941)

Sources	Bact. coli, type I %	I.A.C. group %
Relatively pure water	86-98	2-14
Relatively impure water	37-39	61-63

The differences, he remarked, were due to the greater proportions of Bact. aerogenes, type I, intermediates and irregulars in the impure waters.

As regards faeces, the agreement is general that Bact. coli, type I, by far predominates in the faeces of man and animals. He notes that Hill et al (1929) found Bact. aerogenes and the intermediates to constitute up to 16% of the total coliform bacteria in faeces; that Ruchhoft et al (1931) found only 4 types in faeces:

Bact. coli, type I	83·2%,
Bact. coli, type II	11·5%,
Intermediate, type I	2·0%,
Bact. aerogenes, type I	3·3%.

He notes the results of Gray (1932) and of Bardsley (1938) where aerogenes was found respectively in 37 out of 40, and 61 out of 100, specimens of faeces, and quotes Reedy and Puncochar (1940) as finding Bact. aerogenes or intermediates in 87% of 253 samples of human faeces. These authors proved that the ratio of faecal coli to the citrate-utilisers was most commonly 500:1, and that in 166 samples Bact. aerogenes types together with the intermediates were present in numbers of at least 10,000 per gramme.

He recalls the experiments made on stored faeces (Clemesha, 1912; Jordan, 1926; Pawan, 1931; Gray, 1932; Parr, 1936, 1937, 1938; Raghavachari and Iyer, 1939) and notes that in general they showed that the flora varied from day to day, but that the trend was from a predominance of faecal coli to a predominance of the citrate-utilisers.

As regards urines containing coliform bacteria, Bact. aerogenes appeared to be the prevalent type.

Taylor points out that many of the "coliform bacteria" found on grasses and grains (notably those of Rogers, Clark and Evans, 1915) fermented lactose only at 30°C. and were incapable of growth at 37°C., thereby belonging to other genera, e.g., Erwinia. He notes that there is no evidence that true coliform bacteria can multiply on grasses, etc., except perhaps in silage when

the temperature has approached body heat.

The absence of coliform bacteria from unpolluted soil is not, according to Taylor, sufficiently realised. From the literature it would appear that freshly polluted soil shows B. coli to be prevalent, but that afterwards all types gradually die off, B. coli being the first to disappear and aerogenes and the intermediates surviving considerably longer.

The prevalence of coliform bacteria in tropical waters deemed unpolluted has still to be explained. Taylor thinks that possibly the higher temperature and the decomposition of organic matter may provide conditions permitting some multiplication of the more viable types.

In summarising his review, Taylor stresses that there is no evidence to support the view that Bact. aerogenes and the intermediates live normally on plants or in the soil and not in the intestines of man and other animals. While Bact. coli, type I, is by far the most predominant organism in fresh faeces, the other types also occur in relatively small numbers (but still to the approximate extent of 1,000,000 per gramme). In stored faeces and in faecally contaminated soil and water there is a gradual decline of the coliform inhabitants, Bact. coli disappearing first so that the ratio of 500:1 diminishes and is finally reversed.

Summary and Discussion
(1934-1944)

The investigations conducted up to the year 1935 had shown that by a combination of tests (indole, MR, VP, citrate, and gelatin) the coliform group could be subdivided into a number of subgroups of which one (typical B. coli: indole+, MR+, VP-, citrate-, gelatin-) occurred in faeces in enormous numbers, while only small numbers of any of the other subgroups were usually present therein. The recognition of a test, which could by itself single out true B. coli from the other coliform organisms, was therefore of very considerable advantage, and much of the writing of the period under discussion was directed towards assessing the value of the 44°C. modification of Eijkman's test. That it is adequately specific for typical B. coli has been amply proved in temperate zones where the proportion of anomalous results is so small as to be negligible, especially when it is realised that the coliform group is so interlinked that, no matter what test is used, there will always be some "border-line" or atypically-reacting strains. It is equally evident, however, that in the warmer, and especially the tropical, countries the 44°C. test does not have the same value because a significant proportion of the citrate-utilisers are capable of giving a positive

result. The explanation and remedy for this are not yet forthcoming, though the higher atmospheric temperature has doubtless some connection.

In Great Britain, at least, this new test has been universally acclaimed as the best single test for distinguishing faecal coli, and not only has it almost overthrown all the older differential tests but some workers have even proposed that it should replace the ordinary 37°C. presumptive coliform test for the routine examination of water supplies, etc. There is something to be said in favour of the former view and much against the latter. This will be dealt with in the relevant section. It will be remembered that the citrate test, when it was new, was elevated to an almost similar pinnacle of importance.

While most of the investigators of this period were regarding the coliform group from a sanitary viewpoint, one (Malcolm, 1938) contrived to include in his study the minutiae attaching to individual strains. This object was attained with limited success, for he was finally driven back to exclusively sanitary considerations in his conclusion, but the results of his development of MacConkey's work will be further discussed in the experimental section.

For the rest, the interminable debate on the sanitary significance of "atypical B. coli" continued, and right up to the present day there are at least two schools of thought on the subject. To summarise the facts first: typical B. coli (Bact. coli, type I) is universally agreed to constitute at least 90% of the coliform flora of massed faeces, to predominate in a similar manner in sites subject to recent faecal pollution, and to be generally absent or present only in very small numbers in sites not thus polluted. The other coliform types have been shown to occur only in relatively small numbers in massed faeces and recently polluted sites but to form the large majority of the coliform organisms present in materials such as soil, vegetation, etc., where the likelihood of recent faecal contamination could be excluded. These facts may be said to have received unanimous acceptance.

It is in the interpretation that the difference of opinion arises. One body of workers, notably represented by the Ministry of Health (1934, 1939), Topley and Wilson (1936), and Griffin and Stuart (1940) have expressed the belief that the I.A.C. organisms are indigenous to the soil, etc., where they lead a saprophytic existence, that they thereby become ingested with the food of man and animals, and that their presence in faeces is purely adventitious. The occurrence in water of such types when unaccompanied by faecal coli would thus indicate merely the presence of innocent soil washings. This is reminiscent of the belief held until 1934 by Bardsley. In

all fairness it should be pointed out that the Ministry of Health (1939) did not wholly subscribe to this view, but offered repeated cautions against the summary dismissal of I.A.C. types as altogether "non-faecal" in significance.

The other body of workers (Bardsley, 1934; Parr, 1936, 1939; Malcolm, 1938; Taylor, 1942) variously drew attention to the fact that in strictly unpolluted waters and soils no coliform organisms were to be found at all. They also noted that the I.A.C. coliform types were present in nearly all specimens of faeces, and not always in such relatively small numbers. Taylor (1942) pointed out that the numbers in faeces, while small as compared with those of faecal coli, were nevertheless large when compared with the numbers of I.A.C. strains recoverable from soil, etc. It was therefore decided that all the lactose-fermenting coliform bacteria were really faecal in origin, but that the I.A.C. group survived longer outside the body than faecal coli and could thus be found in remotely polluted sites from which true coli had already disappeared. The conclusions finally derived were that faecal coli signified recent faecal contamination, while the I.A.C. group in the absence of faecal coli indicated more remote but nevertheless faecal contamination.

It is noteworthy that the greater ability of the I.A.C. types to survive in extra-intestinal environments was used by the former school as an argument in favour of the non-faecal origin of such types.

My own sentiments have already received expression with those of Suckling (1943) and I am entirely in sympathy with the views advanced by Bardsley (1934) and her followers. I agree with Taylor (1942) that there is no evidence to support the belief that the I.A.C. types are normal inhabitants of the soil. To my mind the findings all point in the opposite direction. With Taylor, however, I feel that further enquiry is necessary to decide with less ambiguity what period of remoteness is to be inferred in the faecal pollution indicated by the I.A.C. types alone.

The question of evolution forms a basis for much fascinating theorising and is closely bound up with the possibilities of variation, mutation, etc., in the coliform group. As already shown, all the evidence until very recently has suggested that the various coliform types are extremely stable. Up to and including the time of Kulp (1932), indole-production appeared to be the only criterion which was subject to alteration, but even this was hinted at rather than proved. The recent work of Parr (1939), Griffin and Stuart (1940) and others would seem to suggest the relative stability of the indole test, but at the same time discredit the absolute

permanence of all the usual attributes of the coliform bacteria. The acquisition or loss of the power to utilise citrate has occurred through prolonged culture in the soil or laboratory tube respectively. How exceptional were the conditions under which the other changes (shifts) took place is not fully revealed, but, while such variations are no doubt proved facts, it is not suggested that they are the rule. For all practical purposes the coliform types, with the exception of Bact. mutabile (which is a slow lactose-fermenter and therefore on the fringe of the strict group), can be regarded as stable organisms.

With regard to the question of evolution, however, the fact that changes have been proved, in whatever exceptional circumstances, opens up the possibility that all the various types are descendents of one original species. Here again there are divergent views. Griffin and Stuart (1940) select aerogenes as the ultimate forerunner of the group. On their theory, it is the ubiquitous highly-reacting coliform organism which has given rise to more specialised varieties which can only multiply in the bowel. These would seemingly include not only faecal coli but the pathogenic non-lactose-fermenters in addition. The weakness of this supposition lies in the lack of proof that aerogenes is, in fact, a normal soil organism. The other theory, to which I subscribe, is that Bact. coli, type I, constitutes the original species. Nowhere but in the lower bowel and faeces are coliform bacteria found in so great numbers, and coli I normally forms the bulk of these. The bowel is thus the breeding ground and coli I the principal type. It is tempting to speculate that on being removed from its natural habitat faecal coli, in an effort to survive, developed the power to utilise non-protein sources for its supply of carbon and nitrogen such as citrate and uric acid respectively, losing at the same time - by reason of the absence of protein - its power to form indole. In such a manner the relatively small numbers of coli II and Intermediate II might be explained, as compared with the fair numbers of Intermediate I. The gradual acquisition of fermentative powers could equally easily be credited, as soil might have provided carbohydrates but only a minimum of protein. The reversal of the MR and VP reactions - demanding as it does a total change in the metabolic processes - is more difficult to accept, but the fact that it has been produced artificially suggests that it can occur naturally, and in such a way aerogenes I would be derived. Together with these changes would run the development of a capsule as an extra protection against unfavourable conditions (shown in the increasing proportions of capsulated strains as the scale is descended), and with the development of liquefactive powers cloacae would arise. Finally the oxytocus stage would be reached where all fermentable substances can be attacked, gelatin liquefied, and so on.

In all this supposition it is not suggested that these changes occur in the few months that are stated to elapse between the deposition of faeces and the total disappearance of the coliform group therefrom. It is, however, mooted that such a trend has been taking place since the earliest times, for in no other way can the existence of the I.A.C. coliform types be explained.

The absence of quantitative data, lamented by Taylor (1942), precludes informed discussion of these points, and the weakest link in the chain is the vagueness of any observations to show multiplication of any of the coliform types in extra-intestinal distributions. Faecal coli is admittedly unable to multiply under natural external conditions (except as regards milk which really forms a natural culture medium), but the evidence concerning the I.A.C. types is discordant, Taylor (1942) restricting any multiplication to such unusual sites as silage, whereas in the water sphere (Wood, 1919; Thresh, Beale and Suckling, 1933; Ministry of Health, 1939; and Suckling, 1943) the possibility of growth in alkaline waters, in pump packing and leather washers, and in the taps themselves, has been more than suggested.

The different distribution and powers of the I.A.C. groups in the tropics also raise problems which, to my mind, can be solved only by the assumption that some multiplication of the citrate-utilisers can take place in exceptional circumstances. Proved facts are lacking, but no other excuse can be raised for the different coliform standards allowed in the hot countries, and without this hypothesis the continued existence of the I.A.C. types cannot be explained. Until more evidence becomes available, however, it would be unjustifiable and wrong (in this country at least) to advance this theory as the explanation of the presence of the I.A.C. types in most waters.

The experiments of Parr (1936) on stored faeces open up a still wider question, the inclusion of non-lactose-fermenters as part of the coliform group. His findings are of the greatest interest. First the citrate-negative coliform types were dominant. These gradually died out leaving the lactose-fermenting citrate-utilisers as the dominant type. This is as would be expected. Finally, however, all the vigorous lactose-fermenters disappeared leaving only a slow lactose-fermenting type of citrate-utiliser. The argument to include the slow lactose-fermenters is thus very strong. Not only Parr (1939) but also Griffin and Stuart (1940) and others are now following the lead of Mackie (1921) and urging the inclusion, as aberrant members of the coliform group, of the anaerogenes and paracolon classes in addition. Paracolon differs from Bact. coli, type I, practically only in not fermenting lactose. It is in many respects

more closely related than aerogenes to Bact. coli. An-aerogenes can simulate almost any of the usual subgroups. Systematically speaking, therefore, no hard and fast line can reasonably be drawn on lactose fermentation. On sanitary grounds, however, a most definite distinction can be made, for in normal massed faeces the slow lactose-fermenters and other aberrant types occur in even smaller numbers than the citrate-utilisers, while non-lactose-fermenting glucose-fermenters are widespread in nature. The views of Wilson (1928) still hold good that the slow lactose-fermenters have little if any sanitary significance, and for the purpose of water examination the coliform group is best regarded as including only those members which can ferment lactose in 48 hours. I shall try to show that we might reduce the limit to 24 hours without great loss of accuracy.

From the sanitary point of view there would appear to be little advantage (in temperate zones) in carrying differentiation further than to distinguish faecal coli. There is certainly no point in distinguishing aerogenes from cloacae, and the gelatin test can once and for all be given up in routine work. What is required is a quantitative estimation of the true coliform bacteria present and a ready method of determining the proportion of these which are faecal coli. Whether the others are Intermediates or etc. may be of interest but is seldom of use.

The Characters and Distribution of the Coliform Bacteria
GENERAL CONCLUSIONS

The coliform bacteria from the beginning and up to the present day have formed the subject of much fascinating discussion. Regarded from a systematic point of view, they constitute an almost indefinable continuum of closely related types stretching from the saprophytic glucose-fermenters on the one side to the pathogenic non-lactose-fermenters on the other. The only constant characters could be said to be their morphology (non-sporing rods) and their ability to grow over a wide range of temperature, i.e., that they can grow on ordinary laboratory media and in the presence of bile salt at room temperature and at blood heat. There is nothing here to distinguish the group from the glucose-fermenting saprophytes on the one hand or some of the intestinal pathogens on the other. From the sanitary point of view, however, only those members which actively ferment lactose are of importance, and this criterion fortunately provides a convenient means of trimming the rough edges. To the hygienist the coliform group comprises a compact and well-defined collection of organisms (with the above characters) which always ferment glucose and lactose (at least) within 48 hours at 37°C. Within this boundary the distinctions between one strain and another are as

interminable and confusing as those on the outer fringe, but, happily, an unusually and extremely clear division is possible at exactly the required point - between the predominantly and exclusively faecal Bact. coli, type I, and the less prominently faecal and longer-surviving members of the other types of coliform bacteria. A comprehensive term for these types is badly needed. If the word "coliform" had not already been applied to the whole group, it would have served admirably here, and even now there is a chance for "Bact. coliforme" as sufficiently distinct from "coliform bacteria." The distinction would be even better if the old word "colon" were used to replace "coliform", so that we should talk about colon bacteria and the colon group, with the two subgroups Bact. coli and Bact. coliforme. Alternatively, the older word "atypical", which is still alive in Mackie and M^{c}Cartney (1942), could well be revived into general use. The term "I.A.C." is clumsy and does not include Bact. coli, type II, which on statistical evidence is no more significant than any of the citrate-utilising types. If the present terms must be retained as at present used and a new word introduced to cover all the Ministry of Health types except Bact. coli, type I, I would suggest Bact. subcoli or Bact. colirarum as indicating the relatively smaller number of such types in the intestine and faeces, but new names are confusing and as enough old ones are available with slight modifications of meaning there is small justification for introducing further complications. I am no taxonomist and, with these suggestions, will leave the delicate subject of nomenclature.

Despite the intermingled nature of the coliform group, the cumulative findings of many observers have demonstrated a clear division between one class, the faecal Bact. coli, type I, and the remainder of the group. The presence in nature of Bact. coli, type I, always indicates recent faecal pollution, and in this country one might hazard the average period since the pollution occurred to be 6 weeks at most. The remaining coliform types would be distinguished by the invariably negative 44°C. test. They would usually utilise citrate and the few strains which were citrate-negative would be indole-negative, or MR-negative, and/or VP-positive, or gelatin-positive, or would give otherwise anomalous results. All these tests would not of necessity be employed. The presence of such types in water (etc.) in the absence of Bact. coli, type I, would usually indicate the occurrence of faecal pollution at some remote date, say, not less than 6 weeks.

The recognition of these two classes (one homogeneous and the other quite heterogeneous) is all the classification necessary for sanitary purposes. The possible methods of accomplishing this will be discussed in the following section.

The coliform group is still interesting many bacteriologists apart altogether from the sanitary question. Electrolytic conditions, serological (agglutination) reactions, and the effect of bacteriophage are being investigated, and it can safely be forecast that our knowledge of these bacteria will be greatly extended in the coming years.

THE BACTERIOLOGICAL EXAMINATION OF WATER

The rôle of the coliform bacteria

Until the coliform bacteria were recognised and tests elaborated for their detection, the potability of water was assessed mainly on chemical considerations together with such bacteriological aid as could be supplied by enumerating the total number of bacteria, per c.c. of the water, which could grow on laboratory media at room temperature and at body heat.

The advent of tests for the coliform group afforded much better evidence for the assessment of sewage pollution than any of the previous methods of analysis. The importance of the coliform bacteria in this respect may be shortly explained thus:

Apart from such chemical questions as lead solvency, the greatest danger associated with drinking water is the possible conveyance by it of certain pathogenic bacteria. The causative organisms of most of the ordinary infectious diseases, such as scarlatina, diphtheria, measles, whooping cough, etc., are not propagated by water as they are unable to survive therein. The intestinal pathogens, e.g., the bacteria causing enteric fever, bacterial food-poisoning, dysentery and cholera, are, however, capable of surviving for a limited period in water. No definite time-limit can be set for the survival of these bacteria because many factors, e.g., the viability of the particular strain, the temperature and quality of the water, exert different effects. From a study of the literature on the subject and some personal experiments, using the latest tetrathionate methods of isolation, I believe that the period of survival of Bact. typhosum is not more than 4 weeks and is generally much less, e.g., 7-10 days. Direct investigation of water for these pathogens would, if practicable, be the ideal method for assessing the safety of water, but even with the newest procedures (which offer great advances) the labour and expense are too great, and the results hardly reliable enough, for routine use. As these pathogens are usually excreted in the faeces, the problem is tackled indirectly by searching for proof of faecal pollution.

The normal bacterial inhabitants of the intestine are the coliform bacteria, faecal streptococci, and the anaerobic spore-bearing bacteria such as Cl. welchii. Of these the coliform bacteria are by far the most numerous, the faecal streptococci the least viable, and the sporing anaerobes the longest-surviving. Advantage can be taken of all these facts in the examination of water,

but the coliform bacteria by their greater numbers afford the greater delicacy as indicators of pollution. Though they would be useless in the unhappy event of a pure water becoming polluted by the urine of a typhoid urinary carrier, this contingency does not seem, fortunately, to constitute more than a hypothetical danger.

Water is most commonly contaminated by sewage pollution, i.e., by receiving a solution of massed faeces. Even if this solution contains the excrement of a case or carrier of typhoid fever, the odds are that the numbers of typhoid bacteria therein will be greatly inferior to those of the coliform bacteria. As days pass and the pathogens more quickly die out, the ratio of coliforms/pathogens steadily increases. It follows, therefore, that intestinal pathogens will normally be accompanied by much greater numbers of coliform bacteria.

As the period of survival of Bact. coli is longer than that of the pathogens, any water which is naturally free from coli can be assumed to be free from dangerous bacteria. Conversely, any water which contains more than minimal numbers of Bact. coli may also contain some pathogenic bacteria, and the greater the number of Bact. coli present, the greater the likelihood of pathogens to be present, and the more dangerous the water. A water showing the presence of only the coliform bacteria other than Bact. coli can be presumed to be free from pathogens and, therefore, safe for the moment, but the presence of remote faecal pollution has been demonstrated, and the possibility of more dangerous contamination indicated.

This subject has received full discussion in the work of Suckling (1943) where the exceptions to these general rules have been detailed. For absolute safety a drinking water should never show the presence of any coliform bacteria in 100 c.c. Such a high standard, though attained by many of the larger water undertakings, would eliminate many of the rural supplies which have been consumed for many years without ill effects. While, therefore, this the standard for all to aim at, one has to be satisfied in many cases with results which give less absolute assurance. All authorities are agreed that the presence of more than minimal numbers of Bact. coli (1-5 per 100 c.c.) constitutes an indication of danger, but in the case of shallow wells and other sources which cannot always be protected from occasional manurial pollution the presence of larger numbers of the other coliform bacteria is variously allowed. This by reason of the fact that the excreta of animals have not been shown to contain organisms pathogenic to man.

The kind of examination required, therefore, to cover as a routine all the various samples of water sub-

mitted, is one which will allow a rapid enumeration of all the coliform bacteria present per 100 c.c., and at the same time indicate how many of these are Bact. coli. More than this is seldom required and less than this is sometimes sufficient.

The presumptive coliform examination of water

Direct plating methods were originally used but were soon abandoned as permitting too small a volume of water to be examined. Liquid culture methods overcame this difficulty, whereby up to 50 c.c. of water were added to an equal volume of double-strength MacConkey broth (neutral-red bile-salt lactose peptone water), and the mixture was examined after 24-48 hours' incubation at 37°C. for the production of acid and gas (an inverted Durham tube being employed for the demonstration of gas). If after 48 hours the medium did not show these results, the absence of coliform bacteria from the relevant volume of water was proved. The appearance, however, of acid and gas suggested the presence of coliform bacteria and constituted a positive "presumptive coliform" reaction. In this case the tube was then subcultivated to MacConkey agar and red (or acid-forming) colonies developing after incubation were further examined for morphology and lactose-fermentation (by which means the bacteria were confirmed to belong to the coliform group) and the various differential characters necessary to distinguish Bact. coli from the other coliform bacteria.

By inoculating various volumes of water (e.g., one volume of 50 c.c., five of 10 c.c., and five of 1 c.c.) the absence of coliform bacteria from 100 c.c. could be proved, or if coliform bacteria were present an estimate of the approximate number was possible. Until quite recently, the usual procedure was to report the results as in the following examples:

(1)	Coliform bacteria:	Absent from 100 c.c.
(2)	Coliform bacteria:	Present in 100 c.c. Absent from 10 c.c.
	Bact. coli:	Absent from 100 c.c.
(3)	Coliform bacteria:	Present in 10 c.c. Absent from 1 c.c.
	Bact. coli:	Present in 100 c.c. Absent from 10 c.c.
(4)	Coliform bacteria:	Present in 10 c.c. Absent from 1 c.c.
	Bact. coli:	Present in 10 c.c. Absent from 1 c.c.

Result (1) was considered highly satisfactory, (2) reasonably satisfactory in certain cases, (3) of doubtful degree, and (4) definitely unsatisfactory.

Such methods of reporting have, however, become considered to be insufficiently accurate and to be unduly influenced by the necessary sampling error. Statistical tables first elaborated by McCrady (1918) were adopted in a modified form by the Ministry of Health (1934, 1939) so that from a survey of the results it was possible to read off the "probable number" of coliform bacteria per 100 c.c. of the water. Whether this method, at its best, offers any advantage is to my mind doubtful, for the "probable number" obtained is only a statistical one and is itself subject to a very large error, representing as it can anything between double and half the actual number of coliform organisms present. Its accuracy is, therefore, more fictitious than factual, and its statistical nature is difficult for lay councillors and sanitary inspectors to appreciate.

When, added to these disadvantages, it is realised that the "probable numbers" are to be based on the presumptive results alone, i.e., before any definite coliform bacteria have been proved to be present at all, there is considerable doubt left as to what the bacteriologist is actually reporting. This, as Suckling (1943) points out (p. 484), becomes "the probable number of probable coliform bacteria."

If the presumptive reaction were truly specific for coliform bacteria, this last criticism would not apply, but despite the effect of the bile-salt in MacConkey's media in materially reducing the number of false presumptive reactions, there still remain certain bacteria or combinations which can produce acid and gas in the circumstances described. Of these Cl. welchii (see Greer, 1928; Bardsley, 1934) is the principal member, but Suckling (1943) believes that certain bacteria, no one of which in pure culture could exert this effect, can do so by symbiosis in mixed culture.

As the Ministry of Health (1939) used the presumptive results to form a basis for not merely the "probable number" but also a suggested classification for water supplies, it seemed to me important to find out what exact percentage of false presumptive reactions were likely to be met with. For this purpose I analysed some 10,000 consecutive presumptive reactions obtained over a considerable period in the routine laboratory examinations of water samples. The results of this analysis are already shortly described in Suckling (1943) and will be more fully discussed in the experimental section. The final results may be quoted here: 8·65% of the total presumptive reactions were, for one

reason or another spurious; 4·8% were due to anaerobes or other organisms in no way even resembling the coliform group; the remaining 3·85% were due to slow lactose-fermenting bacteria having the coliform morphology, etc. These averages of an extensive investigation are in themselves sufficiently large to be of significance, but it must be emphasised that in the case of particular samples the percentages of false presumptive results might rise as high as 100%. Bardsley (1934) has noted the high percentage of such reactions obtained in the examination of soils. As a parallel I might instance the results obtainable with imperfectly filtered water supplies which have been adequately chlorinated. Such waters may contain quite a number of spore-bearing anaerobes which chlorination is not expected to kill, and may, therefore, though quite free from coliform bacteria, give probable presumptive numbers of 10 to 25, which by the Ministry standards would be unsatisfactory. While admitting that such a water has been imperfectly treated I suggest that to class it with the dangerous waters is ridiculous.

Those authorities who prefer to rely solely upon the presumptive results point out that Cl. welchii and the slow lactose-fermenters are also intestinal inhabitants and have a similar significance to that of the coliform bacteria. My own bias is already obvious, and to this I would reply that until water supplies in this country can be raised to such a standard as to be free from all signs of faecal pollution, I should require, before hazarding an opinion, to have some idea as to whether the faecal pollution discovered related to period distant by 3 weeks, over 6 weeks, or several years (Cl. welchii). In the absence of some such information I should be unable in many cases to avoid condemning a supply which might be quite reasonably satisfactory.

If it were imperative to restrict the examination of water to the presumptive test, some help could be afforded by using discrimination before accepting the presumptive results. The false reactions given by the anaerobes, etc., are often distinctly different from those given by the coliform bacteria. Thus, in the former case, the tube is less strongly acid and the amount of gas almost minimal. Such differences are not, however, invariably detectable, and as plating (with a consequent delay of only 24 hours at most) reveals the spurious results due to the anaerobes, its omission on grounds of expense is not justifiable.

Further investigation.

Sufficient has been said to prove that the 37°C. presumptive test, while providing a convenient initial

stage in the isolation of the coliform bacteria, does not constitute a sufficiently reliable final test. Some further investigation is necessary. The usual procedure to which I have been accustomed is fully described in Suckling (1943) and the alternative methods suggested by the Ministry of Health (1939) have also been discussed therein.

In laboratories where a large number of water samples are received daily, the convenience and accuracy of plating, isolation, confirmation, and the application of differential tests leave nothing to be desired. The loss of time, so greatly apprehended by workers inexperienced in the routine, does not in fact exist. Thus, the great majority of the drinking waters are "negative in 100 c.c." to the presumptive test and require no further investigation for coliform bacteria. In the case of the others, every positive presumptive tube is followed up and checked, wherever this is practicable. In certain cases, where 6 or more tubes have given the presumptive reaction, the best compromise is to take a representative selection for further examination, and, if plating confirms the absence of spurious reactions, such waters can be condemned while the differential tests are proceeding. In this way, all delay is avoided and sufficient is usually known within 48 hours to form a basis for action where speed is essential. The secret of success in this method lies in the fact that a large majority (some 75%) of the true presumptive reactions develop within 16-20 hours of the commencement of incubation.

Where such is the normal routine, the advent of the 44°C. test is acceptable as a useful confirmatory criterion, but its employment is not essential. Where, however, reliance has previously been placed purely on the 37°C. presumptive reaction, the 44°C. test offers very considerable advantage. The best method of combining the two tests is to use them in parallel as presumptive reactions, one for possible coliform bacteria and the other for possible Bact. c oli. In this way both are subject to the anaerobes error but the 44°C. reaction is not given by the slow lactose-fermenters or, in fact, by anything in this country except type I coli (and Irregular II which can be lumped with coli I without significant error). The neatest method of combination I have seen is that of Thornton (1944) of Salisbury, who puts up 55 c.c. of water at each temperature (5 tubes of each of 10 c.c. and 1 c.c.) and calculates after 48 hours the probable number of (a) possible coliform bacteria and (b) faecal coli, per 100 c.c. of the sample.

To adopt this method as a routine in a laboratory with a large water practice would necessitate the provision of a very considerable water-bath accommodation, but this procedure is ideal for the ordinary pathological laboratory which receives only a few water samples each day.

This variation of routine is nevertheless always available to any laboratory which has the requisite apparatus, and can be usefully employed as the circumstances demand. Where the error of the 37°C. test is controlled by the 44°C. test, the combined results can in most cases be accepted without the usual confirmation, but no time has been lost in the process and, where necessary or desirable, the isolation, etc., can be continued in the usual way.

Methods other than those described are in my opinion of very limited value. To apply the citrate test to a mixed culture of unknown organisms is, for example, futile in the extreme, because many organisms outside the coliform group can utilise citrate. Any method which does not include isolation of the bacteria sought must be subject to some degree of fallacy, and the only argument in favour of short-cuts is that frequent short examinations are preferable to less frequent and more elaborate ones.

As so much effort has been directed towards dispensing with the ordinary differential tests, and in view of the observations of Malcolm (1938) on colonial appearances, I was tempted to study whether a combination of presumptive tests together with plating alone would suffice to distinguish (a) true from false presumptive reactions, and (b) faecal coli from the other coliform bacteria. This study was brought to a premature end by the destruction of the laboratory, but the observations are submitted in the experimental section as affording some, if limited, information.

EXPERIMENTAL SECTION

1.

AN ENQUIRY INTO THE ACCURACY OF THE PRESUMPTIVE COLIFORM REACTION.

That the presumptive coliform test is subject to fallacies which render a confirmatory lactose-fermentation test necessary has long been the opinion of Suckling. This view is not, apparently, shared by other authorities. The Ministry of Health (1934, 1939), while meticulously describing various differential tests, omit reference to any need for confirming the fermentation of lactose by the organisms isolated from the presumptive tubes. Mackie and M^{c}Cartney (1942) go even further and state (p. 299): "The fermentation of lactose may be assumed from the presumptive test." While experience has shown this to be largely true in the examination of water, it was considered advisable to enquire what exact percentage error existed over an extensive series of presumptive reactions. For this purpose I scrutinised a lengthy section of our routine water examinations, and traced the fate of some 10,390 consecutive presumptive reactions. These results, reduced for convenience to a common denominator of 10,000, were tabulated in the recent edition of Thresh, Beale and Suckling's "The Examination of Waters and Water Supplies" (Suckling, 1943; Table XIII, p. 478) together with a short commentory. As the table is too elaborate to be typewritten on one page, it is reproduced in 3 sections in Tables 62-64.

The presumptive reactions were obtained in the routine examination of several thousand samples of water, presenting almost every possible variety of type, quality, etc. As the presumptive reactions were the centre of interest no note was taken of the number, type, quality, etc., of the samples. Moreover, as the survey was retrospective, there was necessarily no deviation from the normal routine.

Although the previous section has given some indication, the ordinary routine employed requires brief description. In the case of most drinking waters (which previous prolonged experience had shown to be unpolluted) the following volumes were subjected to the presumptive coliform tests: 50 c.c., five of 10 c.c., one of 1 c.c. and one of 0·1 c.c. Waters whose purity was less certain were allowed 5 quanta of 1 c.c. and of 0·1 c.c.; and in the case of waters expected or suspected to be fairly polluted decimal dilutions were prepared and quanta from 10 c.c. to 0·0001 were tested. In all cases the sample was first of all thoroughly shaken - a most important

preliminary, the omission of which is the most probable cause of the sampling anomalies for which the Ministry of Health make so much allowance and which we very seldom encountered.

All these tubes were incubated for 48 hours at 37°C., being inspected at intervals for the appearance of the presumptive reaction. As a rule, every tube showing acid and sufficient gas was subcultured (as soon as discovered) to MacConkey agar; where, however, a long consecutive series of tubes from one sample showed the presumptive reaction, a representative selection was made, e.g., the 50 c.c., three of the 10 c.c. volumes, and so on. Attention was thus directed but not confined to the smallest positive volumes (cf. Bardsley, 1926, 1934). The agar plates once spread were incubated at 37°C. for 24 hours and then examined. Those showing no growth or only colourless colonies were returned to the incubator for a further period of 24 hours, and if the appearances remained the same they were then discarded. These are recorded in Table 62 under "anaerobes" (no growth whatever) and "white colonies and red cocci" (only the non-lactose-fermenting bacilli or the pin-head deep red colonies of the faecal streptococci, etc.). In most cases, however, there was within 24 hours a collection of possible coliform colonies (whose appearances are more fully described later). From these, in each case, the colony most likely to be Bact. coli, type I, was picked, tested for morphology and Gram-staining, and subcultured into peptone water (for the indole test), glucose-phosphate medium (for the MR test), Koser's citrate medium, and MacConkey lactose broth (for the confirmatory test). Where plates presented no colony resembling that of Bact. coli, the colony most commonly represented was selected.

These tubes were incubated for 24 hours at 37°C., by which time the great majority of the lactose tubes showed adequate fermentation. In such cases, the differential tests were performed and the results noted. In all other cases, the tubes were returned to the incubator and tested 24 hours later, the presence or absence of lactose-fermentation being carefully noted, for the non-fermenters - whatever their differential characters - were "written off" as of insignificant sanitary importance, though records of the reactions were kept for reference purposes.

It had long been the habit in the laboratory to regard early presumptive reactions as more ominous than later ones, i.e., as more likely to reveal Bact. coli, type I. The presumptive results were thus always carefully recorded as "first day", i.e. as occurring within 24 hours of the commencement of incubation, or "second day", i.e., occurring between 24 and 48 hours after the commencement of incubation. This is reflected in the

TABLE 62.

The results of differentiating 10,000 presumptive coliform reactions obtained during routine examination.

A.

Showing the false reactions revealed by plating and the percentages of types obtained without using the confirmatory lactose-fermentation test.

DIFFERENTIAL CLASSIFICATION		PRESUMPTIVE COLIFORM REACTIONS. (Acid and sufficient gas in MacConkey Broth at 37°C.)						
		First day		Second day		Total		
		No.	%	No.	%	No.	%	%
Colony-confirmed Presumptive Coliform Reactions.	Bact. coli, type I.	6369	83·3	770	32·7	7139	71·4	75·0
	Bact. coli, type II.	229	3·0	255	10·8	484	4·8	5·0
	Intermediate, type I.	416	5·4	504	21·5	920	9·2	9·7
	Intermediate, type II.	138	1·8	118	5·0	256	2·6	2·7
	Bact. aerog., type I, and Bact. cloacae.	300	3·9	247	10·5	547	5·5	5·7
	Bact. aerog., type II.	42	0·6	41	1·7	83	0·8	0·9
	Irregulars.	33	0·4	58	2·5	91	0·9	1·0
	TOTALS	7527	98·4	1993	84·7	9520	95·2	100·0
False presumptive Reactions.	"White colonies" and red cocci.	73	1·0	274	11·6	347	3·5	
	"Anaerobes"	45	0·6	88	3·7	133	1·3	
TOTALS		7645	100·0	2355	100·0	10,000	100·0	

TABLE 63.

Showing the numbers and percentages of non-lactose-fermenting (slow-lactose-fermenting) coliform bacteria obtained by applying the confirmatory lactose-fermentation test to 9520 colony-confirmed coliform bacteria arising from 10,000 simple presumptive reactions.

B.

DIFFERENTIAL CLASSIFICATION	"NON-LACTOSE-FERMENTERS" (Gas not produced by pure cultures in MacConkey Broth in 48 hours, 37°C.)					
	First day		Second day		Total	
	No.	%	No.	%	No.	%
Bact. coli, type I.	1	0·016	15	2·0	16	0·2
Bact. coli, type II.	41	17·8	86	33·7	127	26·2
Intermediate, type I.	44	10·6	75	14·9	119	12·9
Intermediate, type II.	7	5·4	7	5·9	14	5·5
Bact. aerog., type I, and Bact. cloacae.	9	0·3	33	13·4	42	7·7
Bact. aerog., type II.	1	2·4	4	9·7	5	6·0
Irregulars	12	36·4	50	86·2	62	68·2
TOTALS	115	1·5	270	13·5	385	4·0

Note: The above percentages are obtained by comparing the adjacent number with the corresponding number in Table 62. The columns headed "First day" and "Second day" refer in both tables (and also in Table 64) to the original presumptive coliform reactions. Thus, presumptive reactions appearing within 24 hours and the organisms isolated therefrom are found in the "First day" columns.

TABLE 64.

Showing the number of cases in which 10,000 presumptive coliform reactions (9520 plate-confirmed colonies) were due to true coliform bacteria as determined by confirmatory and differential tests. Showing also the percentages of the various types found in the search for Bact. coli, type I.

DIFFERENTIAL CLASSIFICATION	TRUE COLIFORM BACTERIA (Obtained by deducting the numbers in Table 63 from the corresponding numbers in Table 62, i.e., "A" minus "B")					
	First day		Second day		Total	
	No.	%	No.	%	No.	%
Bact. coli, type I.	6368	86·0	755	43·8	7123	78·0
Bact. coli, type II.	188	2·5	169	9·8	357	3·9
Intermediate, type I.	372	5·0	429	24·9	801	8·8
Intermediate, type II.	131	1·7	111	6·4	242	2·6
Bact. aerog., type I, and Bact. cloacae	291	3·9	214	12·4	505	5·5
Bact. aerog., type II.	41	0·6	37	2·2	78	0·9
Irregulars	21	0·3	8	0·5	29	0·3
TOTALS	7412	100·0	1723	100·0	9135	100·0

Note: Tables 62, 63 and 64 should be placed alongside one another, as indicated by the dotted lines. Table 64 may usefully be compared with Table 38 (p. 66) where the results obtained by Bardsley (1934) are similarly tabulated.

tables where the "first day" and "second day" presumptive reactions and their respective end-results are segregated throughout.

Reference to Table 62 reveals that of the 10,000 original presumptive reactions, 7645 occurred in the first 24 hours and 2355 in the 24-48 hours' period. The plating technique alone revealed 480 (4·8%) of the total reactions to be spurious and due to anaerobes or a mixture of non-lactose-fermenting organisms, and it is noteworthy that only 118 (or less than one-quarter) of the 480 false presumptive reactions occurred in the first 24 hours. Looked at in another way, of the 7645 "first day" presumptive reactions only 1·6% were false in this respect, whereas 15·3% of the 2355 "second day" reactions were due to spurious organisms. In this table also it is shown that Bact. coli, type I, was responsible for 83·3% of the "first day" reactions, only 32·7% of the "second day" reactions, and 71·4% of the total 10,000 or 75% of the 9520 colony-confirmed reactions. The laboratory belief was, therefore, amply justified, and another "superstition" given support: that a water which fails to produce any presumptive reactions within 24 hours has a good chance of being free from Bact. coli, and vice versa.

As the heading points out, all the observations in Table 62 are made without any reference to the confirmatory lactose test. This is taken into consideration in Table 63 where it is shown that by failing to pass this test 385 (3·85% of the original 10,000, 4% of the 9520 colony-confirmed, reactions) have had to be excluded. The percentages in this table are based on the corresponding numbers of presumptive reactions (in Table 62) from which each group of non-lactose-fermenters arises. Most noteworthy is the fact that 6369 "first day" reactions which resulted in coli I yielded only one slow-lactose-fermenter. Regarding the total coli I column, one sees that in this category of 7139 organisms satisfying the differential criteria only 16 (0·2%) failed in the lactose test. So far, therefore, as coli I is concerned, the confirmatory test could be omitted.

The position among all the other types is materially different, however, where not less than 5·5% of the total of any one type proved to be slow lactose-fermenters, and much higher percentages than this were obtained, e.g., 26·2% of coli II, and 68·2% of the Irregulars. Again, the differences between the "first day" and "second day" results are notable. Of the 385 non-lactose-fermenters only 115 (1·5%) arose from "first day" reactions, whereas 270 (13·5%) were derived from the "second day" presumptive tubes. While it is always possible that some unpicked colony represented the organism responsible for the original presumptive reaction, this does not remove the need for applying a

confirmatory test to the selected organism.

As altogether 480 + 385, or 865 (8·65%) of the total 10,000 presumptive reactions have been discredited by further tests, the need for plating and for a confirmatory lactose test is, in my opinion, established. The one criterion which could with reasonable justification be assumed is the morphology. I have never yet seen an acceptably "coliform" colony on MacConkey agar which did not yield Gram-negative non-sporing rods.

Turning now to Table 64 (from which all organisms outside the strict coliform group have been excluded) we notice that the total in the "first day" column (7412) represents about 97% of the original gross total of "first day" reactions (7645) and 98·5% of the "first day" colony-confirmed reactions (7527). Moreover, Bact. coli, type I, accounts for 83·3% (6368) of these gross "first day" reactions (7645) and 84·4% of the "first day" colony-confirmed reactions. Hence the conclusion (already quoted in Suckling, 1943) that: "first-day presumptive reactions may be regarded as 97% 'true', and as containing at least 80% of Bact. coli, type I."

The position is considerably different in the case of the "second day" reactions for here the total of true organisms obtained (1723) represents only 73·2% of the gross "second day" total (2355) and 86·5% of the colony-confirmed total (1993), while Bact. coli, type I (755), accounts for only 32% of the gross and 37·8% of the colony-confirmed total.

If Bact. coli, type I, were the only significant coliform type, the logical conclusion would be to concentrate on 24-hour results, in which case the subsequent plating, differential and confirmatory tests might be omitted, and the standards slightly raised to allow for the 12% loss through neglect of the 48-hour results. It is still possible that a greater degree of accuracy might be obtained in 24 hours by using the 44°C. test in place of the 37°C. test. (This subject is at present being investigated by Thornton at Salisbury.) Sufficient, however, has been already said to show that the presumption involved in either of these cases is unsafe, even in this country. The other coliform types provide a guide to some cases of pollution from which coli has already disappeared. Hence they must not be neglected. Once they have been included in the examination they bring with them a large proportion of delayed (37°C.) presumptive and false presumptive reactions, and necessitate all the "trials" of confirmation and differentiation.

Table 64 also shows the various proportions of the "non-coli-I" types found during the search in water for Bact. coli, type I. The ultimate figure for coli I (78%)

is not, perhaps, very impressive, but it must be remembered that there was a fair number of plates which presented no obviously coli colonies, and that mucoid or otherwise "non-coli-I" colonies had perforce to be selected. The figure is, nevertheless, considerably greater than that obtained (68%) by Bardsley (1934).

The proportions in which the various types were obtained are not suggested to give any indication of the proportions in which they occurred in the massed samples, i.e., in water generally. In the first place, it is acknowledged (Wilson et al., 1935) that the preliminary enrichment in MacConkey's medium favours coli I at the expense of the other types (which is, of course, ideal for the purposes of the examination). Secondly, the "non-coli" colonies were avoided as far as possible. Hence, obvious aerogenes-type colonies would never be picked while there was any alternative available. The percentages of all non-coli types are, therefore, understated, and this is particularly so in the case of the MR-negative types which so commonly form mucoid colonies. As will be shown later, a proportion of the Intermediate colonies very closely resemble those of coli; therefore the Intermediates have a certain share in the relative emphasis conferred on Bact. coli. Nevertheless, there is practically no method which can be combined with the routine examination and which will at the same time give a fair picture of the coliform types as they actually occur. The percentages shown are of interest as being remarkably constant, and as showing the relative proportions of the other types recovered during the search for coli I. It is quite possible that they are as much subject to the technique of isolation as to the proportions of types originally present.

2.

A DETAILED STUDY OF SOME COLIFORM STRAINS, WITH PARTICULAR REFERENCE TO THE POSSIBLE VALUE OF COLONIAL APPEARANCES.

In view of the observations of Malcolm (1938), many of which I desired to check, and in the hope of proving that colonial appearances might afford some reliable assistance in distinguishing coli I from the other coliform types, I determined to subject a number of coliform strains (obtained at different times from various waters) to an exhaustive scrutiny. A total of only 43 organisms had been fully tested before the laboratory was destroyed, and the emergency premises to which we removed in the suburbs did not permit continuance of the investigation. The observations, though therefore limited in scope, are not, however, devoid of interest.

The organisms were plated on MacConkey agar from

the confirmatory lactose tubes. Their differential (indole, MR and citrate) criteria were thus already known. These were re-tested several times, after periods ranging from 24 to 96 hours, and though the VP reaction was also included the results remained constant and provided no anomalies. The small numbers may have accounted for this, or it may have been the result of selection. The only warrantable conclusion is that the Irregulars were not the subject of the present study.

Each strain was verified to be a pure culture of Gram-negative, non-sporing rods and was tested for the following characters:-

(1) motility - after 12-24 hours' growth in nutrient broth at room temperature.

(2) encapsulation - by Muir's method of staining (see Muir and Ritchie, 1937), and by the relief method of Howie and Kirkpatrick (1934) as recommended by Malcolm (1938).

(3) colonial appearances on nutrient agar.

(4) colonial appearances on MacConkey agar (neutral-red bile-salt lactose peptone-water agar). It is important that the bile-salt be pure sodium taurocholate and not the commercial variety, as with the latter a purplish haze surrounds most of the colonies and obscures their appearances.

(5) liquefaction of gelatin - stab cultures in nutrient gelatin grown at 20°C. for 6 weeks.

(6) effect on litmus milk - 3 days' incubation at 37°C.

(7) fermentation of the following carbohydrate media:-

glucose	inositol
galactose	adonitol
lactose	sucrose
maltose	raffinose
rhamnose	salicin
arabinose	dulcite
xylose	inulin
mannitol	
sorbitol	
dextrin	

Incubation at 37°C. for 3 days was allowed for the fermentation tests to enable doubtful 48-hour results to be confirmed or rejected. This extension was only required in 2 cases, fermention being at the end of 48 hours either well advanced or not commenced. The delayed reactions both occurred in a salicin tube.

Preliminary tests showed that all the carbohydrates in the first column above were invariably fermented. Of these, only lactose was included in the further study.

Litmus milk, though not invariably clotted in the rather short time allowed, was also discontinued as offering no detectable help in classification.

In the preliminary experiments (which embraced all the Ministry of Health types) the carbohydrate media were prepared without indicator, and the pH attained after 48 hours was measured by mixing a drop of the culture on a white tile with a drop of various close-range pH indicators. Where fermentation had not occurred the pH was found to be between 7·0 and 7·5, generally 7·5. In the presence of fermentation, the pH varied between 5·7 and 4·0 and was usually between 5·0 and 4·5. No distinction was shown between one group of organisms and another. The variations appeared to depend, in fact, more upon the particular carbohydrate than upon the organism causing the fermentation. This procedure was therefore also dropped, the usual neutral-red being included in future batches of fermentation media.

Throughout the investigation the volume of gas evolved was measured after 24, 48 and 72 hours. Greater volumes were more often produced by the MR-negative than the MR-positive types but there were exceptions on both sides and no decisive help could be demonstrated in this criterion.

The final results are abstracted in Table 65 where it can be seen that the 43 organisms presented only 27 different varieties. The duplicates, it need hardly be observed, would have been discarded if they had been obtained from the same source. Of the 27 varieties, all but 5 could (disregarding the citrate and salicin reactions) be identified with 17 of MacConkey's strains. It will also be noticed that not one inulin-fermenter was found.

The results will now be considered in respect of each Ministry of Health sub-group.

Bact. coli, type I.

Of the total organisms selected, 14 were Bact. coli, type I, according to the indole, MR, VP and citrate - or "Imvic" - reactions. All failed to liquefy gelatin in 6 weeks, and all failed to ferment inositol and inulin. All but two produced the 'typical' non-mucoid colony both on nutrient and MacConkey agar. In these cases, the usual appearance on MacConkey agar (after 24 hours) was a discrete, circular, convex but very slightly raised, non-mucoid colony of about 2-3 mm. in diameter. The central zone of the colony was red and very gradually shaded off to a pink periphery. In a few cases the periphery was paler, and the central zone smaller or more distinctly demarcated. Also in a few cases there appeared among the "typical" colonies an occasional granular (R) colony. This was definitely due to the same organ-

TABLE 65.

Characters of the coliform bacteria isolated.

Serial Number	Indole	MR reaction	VP reaction	Citrate	Motility	Encapsulation	Mucoid colony	Gelatin (weeks)	Inositol	Adonitol	Sucrose	Raffinose	Salicin	Dulcitol	Inulin	Lactose	Name (if any)	MacConkey No.	Variety
1	+	+	-	-	+	-	-	-	-	-	-	-	+	+	-	+	B. coli communis	34	a
2	(coli I)				+	-	-	-	-	-	-	-	+	+	-	+	B. coli communis	34	a
3					-	-	-	-	-	-	+	+	+	-	-	+	B. vesiculosus	5	b
4					-	-	-	-	-	-	+	+	+	+	-	+	B. neapolitanus	72	c
5					+	-	-	-	-	-	+	+	+	+	-	+	B. coli communior	71	d
6					+	-	-	-	-	-	+	+	+	+	-	+	B. coli communior	71	d
7					+	-	-	-	-	-	+	+	-	+	-	+	B. coli communior	71	e
8					+	-	-	-	-	-	+	+	-	+	-	+	B. coli communior	71	e
9					+	-	-	-	-	-	+	+	-	+	-	+	B. coli communior	71	e
10					+	-	-	-	-	+	+	+	-	-	-	+		100	f
11					+	-	-	-	-	+	-	-	-	-	-	+		1	g
12					+	-	-	-	-	-	-	-	-	-	-	+	B. grünthal	4	h
13					-	+	-	-	-	-	-	-	-	-	-	+	B. vesiculosus	5	i
14					-	+	-	-	-	-	-	-	-	-	-	+	B. vesiculosus	5	i
15	-	+	-	-	+	-	-	-	-	-	+	+	-	-	-	+		109	j
16	(coli II)				+	-	-	-	-	-	+	+	-	-	-	+		109	j
17					+	+	+	-	-	-	-	-	+	-	-	+	B. coli mutabilis	8	k
18	-	+	-	+	+	-	-	-	-	-	+	+	-	-	-	+		109	l
19	(Inter. I)				+	-	-	-	-	-	+	+	-	+	-	+		74	m
20					+	-	-	-	-	-	+	+	-	+	-	+		74	m
21					+	-	-	3	-	-	+	+	+	+	-	+		-	n
22					+	-	-	1	-	-	+	+	+	-	-	+		-	o
23					+	-	-	6	-	-	-	-	+	+	-	+		-	p
24					+	-	-	-	+	+	+	+	+	-	-	+	B. friedländeri	68	q
25	+	+	-	+	-	+	+	-	+	+	+	+	+	-	-	+		101	r
	(Inter. II)																		
26	-	-	+	+	+	-	+	3	-	-	+	+	-	-	-	+	B. cloacae	108	s
27	(cloacae)				+	+	+	6	-	-	+	+	-	-	-	+	B. cloacae	108	s
28					+	+	+	3	-	-	+	+	-	-	-	+	B. cloacae	108	s
29					+	+	+	3	-	-	+	+	+	-	-	+	B. cloacae	108	t
30					+	-	+	3	-	-	+	+	+	-	-	+	B. cloacae	108	t
31					+	-	+	6	-	-	+	+	+	-	-	+	B. cloacae	108	t
32					+	-	+	2	+	-	+	+	+	-	-	+		102	u
33					+	-	+	3	-	-	+	+	+	-	-	+	B. cloacae	108	t
34	-	-	+	+	-	+	+	-	+	+	+	+	+	+	-	+		67	v
35	(aerog. I)				-	+	+	-	+	+	+	+	+	+	-	+		67	v
36					-	+	+	-	+	+	+	+	+	-	-	+	B. lactis aerogenes	103	w
37					-	+	+	-	+	-	+	+	+	-	-	+		-	x
38					-	+	+	-	+	-	+	+	+	+	-	+		75	y
39	+	-	+	+	-	+	-	-	+	+	+	+	+	+	-	+		-	z
40	(aerog. II)				-	+	+	-	+	+	+	+	+	+	-	+		-	z
41					-	+	+	3	+	+	+	+	+	-	-	+		-	z'
42					-	+	+	6	+	+	+	+	+	-	-	+		-	z'
43					-	+	+	6	+	+	+	+	+	-	-	+		-	z'

ism, and on such occasions the nutrient agar plates showed similar growths which no doubt correspond to the "vine-leaf" appearance described by the earlier writers as occurring on gelatin slopes. This granular growth is absolutely specific for coli I, as no other type of coliform organism simulates the appearance sufficiently closely.

Of the 12 organisms which produced these "typical" colonies, all were non-capsulated and all but two were motile. Adonitol was fermented by 2 strains, sucrose by 7, raffinose by the same 7, salicin by 6, and dulcitol by 8. Even among such small numbers there was an evident lack of correlation between the fermentation reactions (except, of course, for sucrose and raffinose) and these criteria were thus confirmed to be useful only for the separation of individual strains.

The 2 "aberrant" coli I strains were notably distinct by failing to ferment any of the "differential" sugars (column 2, page 115) and by producing mucoid and confluent colonies on both nutrient and MacConkey agar. Both were non-motile and one was distinctly capsulated. The colonies, before coalescing, attained a diameter of 3-4 mm. and retained a pink colour with a reddish (not yellowish) centre.

Bact. coli, type II.

Of the 3 strains giving the "Imvic" reactions of Bact. coli, type II, all were gelatin-negative and failed to ferment inositol, adonitol, dulcite and inulin. Two fermented sucrose and raffinose, and were motile, non-capsulated organisms giving discrete non-mucoid colonies on MacConkey agar very similar to those of coli I. The third strain fermented salicin, was non-motile, capsulated and produced mucoid, confluent colonies with a reddish-pink centre and a pink opalescent ring-periphery. Though this organism conformed in its reactions to that named "B. coli mutabilis", it did not show any of the variation that could have been expected on subculture.

Intermediate, type I.

Seven of the strains gave the "Imvic" reactions of this sub-group. All were non-fermenters of inulin, and 6 did not attack inositol and adonitol. Of these 6, 4 were motile, non-capsulated forms which produced discrete non-mucoid colonies practically indistinguishable from the "typical" coli-I appearance. One of these liquefied gelatin in 18 days. The 3 organisms which gave rise to mucoid, confluent colonies were non-motile. In no case could a definite capsule be demonstrated, but in all 3 cases the colony showed a deep-red pin-head centre sharply marked off from a white, mucoid periphery. Inositol was fermented by one of these and unattacked by the others.

Intermediate, type II.

The only strain representing this type was a capsulated, non-motile inositol-fermenter which produced confluent mucoid colonies of which nearly the whole was filled by the large red centre. This appearance had been frequently noted in routine work.

Bact. cloacae.

Eight strains belonging to this sub-group were investigated. All were motile non-fermenters of inulin, dulcitol and adonitol. Inositol was fermented in one case (which was anomalous in not at the same time fermenting adonitol). Sucrose and raffinose were fermented in all cases, and salicin in 5. Seven of the strains produced mucoid colonies which soon became confluent. Before coalescing, each colony presented a usually eccentric, red-spot (1-2 mm.) centre sharply demarcated from a pale (almost colourless) mucoid periphery. As coalescence occurred, these spot-centres persisted giving the appearances described by MacConkey (1905). Definite capsules were clearly demonstrable in only 3 cases. Gelatin was liquefied in all cases, usually by the end of 3 weeks.

The remaining strain (also a gelatin-liquefier) produced discrete, non-mucoid colonies, somewhat similar to those of coli I but with a whiter periphery.

Bact. aerogenes, type I.

The five strains conforming to this type were all non-motile, capsulated organisms, giving rise to mucoid colonies and fermenting inositol, sucrose, raffinose and salicin. Adonitol was anomalously not fermented by two strains; dulcitol was fermented by 3, and inulin by none. Gelatin was not liquefied. The colony originally showed a pink-red centre with a pale mucoid translucent periphery. Confluence soon occurred, and after 48 hours the pink colour gradually changed to a yellowish brown. All the MR-negative organisms showed this colour change to some degree.

Bact. aerogenes, type II.

Of the 5 strains belonging to this type, 3 liquefied gelatin and 2 did not; 4 gave mucoid colonies and all 5 fermented inositol and adonitol, sucrose, raffinose and salicin. Inulin was fermented by none, and dulcitol by 2. All were non-motile and 3 showed definite capsules. One of these gave definitely non-mucoid colonies, which were nevertheless clearly distinguishable from those typical of coli I.

Conclusions.

Even the small numbers embraced by this study show that not one of the criteria investigated gives a sharp dividing line throughout. Except for the absolute correlation between the fermentation of sucrose and that of raffinose (explained by Browne, 1914), there are exceptions to every general rule which might be formulated. While the findings of Malcolm (1938) have been confirmed in general, no absolute correlation was found between inositol and adonitol, either as regards fermentation or non-fermentation. Nor was there any very close association between inositol-fermentation and the production of mucoid colonies. Despite these qualifications, it would seem that inositol is a better criterion than indole if the Intermediates must be subdivided. This suggestion is amplified in Suckling (1943).

Colony appearances provide only a rough guide to the differential characters. A certain "typical" discrete, non-mucoid colony showing a gradual shading from red centre to pink periphery restricts the organism to Bact. coli, type I, inositol-negative Intermediate, and a few Bact. coli, type II; but a mucoid colony though uncommon in the case of coli I can be produced by organisms belonging to any of the sub-groups. Where the "typical" non-mucoid colony is obtained in routine examinations the presumption of faecal coli, though liable to error, is fairly strong.

The examination for encapsulation is far more cumbersome than the results merit. In quite a number of cases, even by using several methods, only a doubtful conclusion can be reached. Moreover, definitely capsulated strains (which, except for cloacae, are usually non-motile), although normally producing mucoid colonies, are capable at times of giving rise to non-mucoid appearances; and the non-capsulated (and usually motile) forms do not invariably produce non-mucoid colonies. It can be hazarded that capsule-formation is probably the most variable characteristic of the coliform group. Growth in milk medium has long been known to favour the formation of capsules in the coliform bacteria, and it is more than likely that the production of a capsule is, certainly among the non-coli-I types, a temporary attribute dependent upon the prevailing circumstances.

While it is to be hoped that further study may elicit some unexceptionable facts about the coliform group, this is highly unlikely. From whatever angle the coliform bacteria are approached they seem to prove themselves extremely heterogeneous. Even the best-marked types (coli I and cloacae) present some exceptional members, and it would seem unwise to attempt more than a sanitary "rough-sorting" into the predominantly faecal

Bact. coli, type I, and the other less prominently faecal types. For this purpose the simplest method is certainly the application to proved coliform colonies of the 37°C. and 44°C. lactose-fermentation tests, but until more observations are made it is safer not to place absolute reliance upon one (the 44°C.) test but to include as parallel tests the indole, MR, (VP) and citrate reactions and possibly inositol-fermentation. For routine water analysis the method of choice is, to my mind, the ordinary 37°C. presumptive test followed by plating and the confirmatory 37°C. test with the 44°C. test in parallel as a close enough guide to coli I. These tests could always be further elaborated at the discretion of the examiner.

SUMMARY.

(1) The literature has been perused in a roughly chronological order, emphasis having been given to those workers whose observations are most generally important or have most bearing upon the title of this thesis. The gradual replacement of fermentation reactions by other tests (indole, VP, MR, citrate, 44°C.) for the purposes of classification has been shown to result from a desire to classify on a sanitary rather than on a purely systematic basis. The difficulties in this latter respect have been expounded, and the coliform bacteria (when regarded systematically) have been shown to consist of a heterogeneous collection of strains which cannot be sharply distinguished from the intestinal pathogens, on the one hand, or from the glucose-fermenting saprophytes on the other. Those members which are most commonly present in faeces, however, and which have, therefore, paramount sanitary significance, are characterised by their activity in fermenting lactose, and this criterion is used by the hygienists to form a restricted coliform group as a basis for indicating faecal pollution. This group is fairly clearly subdivisible into two classes: (a) the "faecal" or "typical" Bact. coli (reasonably homogeneous) which is highly predominant in fresh faeces and soon dies once outside the body; and (b) a very heterogeneous conglomeration (for which there is as yet no comprehensive term) whose members occur in relatively small numbers in fresh faeces and are capable of longer life in external circumstances.

(2) The significance of the presence of these coliform types in water and the best means for their detection therein are discussed.

(3) In view of the present trend to utilise "short-cuts" in the coliform examination of water, and particularly to base sanitary standards upon the results of "presumptive coliform" reactions, the inherent fallacies of such procedures are explored, and attention drawn to the need for some confirmatory tests. While full differentiation is probably not necessary in this country, the minimum addition to the 37°C. presumptive test would seem to be a parallel 44°C. test or subsequent plating on MacConkey agar.

(4) A small-scale investigation has shown that the presumptive test followed by plating alone is hardly adequate to distinguish faecal coli, though nevertheless possessing considerable advantages over the generally inadequate simple presumptive test.

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